Title: Mutant Viruses

Field of the Invention

5   The present invention relates to mutant herpes simplex
     viruses wherein the herpes simplex virus genome comprises
     nucleic acid encoding a nitroreductase.

Background to the Invention

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Herpes simplex virus

The herpes simplex virus (HSV) genome comprises two
covalently linked segments, designated long (L) and short

15   (S). Each segment contains a unique sequence flanked by a
     pair of inverted terminal repeat sequences. The long
     repeat (RL or R_L) and the short repeat (RS or R_S) are
     distinct.

20   The HSV ICP34.5 (also γ34.5) gene, which has been
     extensively studied[1,6,7,8], has been sequenced in HSV-1
     strains F[9] and syn17+[3] and in HSV-2 strain HG52[4]. One copy
     of the ICP34.5 gene is located within each of the RL
     repeat regions. Mutants inactivating both copies of the

25   ICP34.5 gene (i.e. null mutants), e.g. HSV-1 strain 17
     mutant 1716[2] (HSV 1716) or the mutants R3616 or R4009 in
     strain F[5], are known to lack neurovirulence, i.e. be a
     virulent, and have utility as both gene delivery vectors
     or in the treatment of tumours by oncolysis. HSV-1 strain

30   17 mutant 1716 has a 759bp deletion in each copy of the
     ICP34.5 gene located within the BamHI s restriction
     fragment of each RL repeat.

ICP34.5 null mutants such as HSV1716 are, in effect,
first-generation oncolytic viruses. Most tumours exhibit
individual characteristics and the ability of a broad
spectrum first generation oncolytic virus to replicate in
or provide an effective treatment for all tumour types is
not guaranteed.

HSV 1716 is described in EP 0571410 and WO 92/13943 and
has been deposited on 28 January 1992 at the European
Collection of Animal Cell Cultures, Vaccine Research and
Production Laboratories, Public Health Laboratory
Services, Porton Down, Salisbury, Wiltshire, SP4 0JG,
United Kingdom under accession number V92012803 in
accordance with the provisions of the Budapest Treaty on
the International Recognition of the Deposit of
Microorganisms for the Purposes of Patent Procedure
(herein referred to as the 'Budapest Treaty').

Nitroreductase prodrug activation

Enzyme prodrug therapy is based on the enzymatic
activation of a non toxic or low toxicity prodrug to a
form that is considerably more cytotoxic. The activation
may involve enzymatic reduction of the prodrug to a
cytotoxic reduced form.

The E.coli nitroreductase enzyme (NTR) has been proposed
for use in gene-directed enzyme prodrug therapy (GDEPT)
as an activating enzyme for nitroaromatic prodrugs of the
dinitrobenzamide class[16]. E.coli NTR is a homodimeric
enzyme with two active sites and is the oxygen

insensitive enzyme from E.coli (the nfsB gene product).
It has the ability to reduce a wide range of nitro-
containing compounds such as nitrofurazone (to the
hydroxylamines) and quinones such as menadione (to the
quinols). It is specifically inhibited by the
irreversible inhibitor dicoumarol.

The ability of NTR to reduce aromatic nitro groups to the
corresponding hydroxylamine (and possibly amine)
derivatives has been proposed for cancer chemotherapy
mainly with the dinitrobenzamide class of prodrugs. The
5-aziridin-1-yl-2,4-dinitrobenzamide CB1954 (CAS Registry
number 21919-05-1)is one such prodrug which has been
studied as a prodrug for GDEPT with NTR[16].

Cyclic and acyclic nitroaryl phosphoroamide mustard
analogues have also been shown to be activated by E.coli
NTR[17]. The acyclic 4-nitrobenzyl phosphoramide mustard
showed 167,500x selective cytotoxicity toward
nitroreductase-expressing V79 cells with an IC_{50} as low as
0.4nM which is about 100x more active and 27x more
selective than CB1954.

Recombinant adenovirus and recombinant retrovirus[10]
expressing nitroreductase have been constructed for use
with the prodrug CB1954 with the intention of providing a
treatment for cancer. The recombinant virus is not
oncolytic and relies on gene directed enzyme-prodrug
therapy to achieve tumour cell kill.

Summary of the Invention

The inventors have determined that herpes simplex virus
having an inactivating mutation in the RL1 locus, more
specifically a mutation which inactivates the function of
the ICP34.5 gene product, such that the herpes simplex
virus does not produce a functional ICP34.5 gene product
and is non-neurovirulent, can be used in the delivery to
a cell of a gene encoding a gene product useful in
targeted tumour therapy.

The inventors have provided a novel second generation
oncolytic mutant HSV. The genome of this mutant HSV
comprises the heterologous (i.e. non-HSV originating)
E.coli nitroreductase protein coding sequence inserted at
one or each ICP34.5 locus, disrupting the ICP34.5 protein
coding sequence such that the ICP34.5 gene is non-
functional and cannot express a functional ICP34.5 gene
product. The generated HSV is capable of expressing the
E.coli nitroreductase gene product under control of the
inserted promoter. This virus thus has the oncolytic
activity of HSV strain 17 mutant 1716 and can be used in
gene directed enzyme-prodrug therapy (GDEPT) and has
shown significantly enhanced tumour cell killing in vitro
and in vivo when used with the prodrug CB1954. The mutant
virus is designated HSV1716/CMV-NTR/GFP (also called
HSV1790).

HSV1716/CMV-NTR/GFP is an engineered herpes simplex virus
ICP34.5 null mutant which expresses the nitroreductase
(NTR) gene. This virus provides for enhanced virus
induced tumour cytotoxicity. It combines NTR transgene

delivery and CB1954 prodrug treatment with the
proliferation-specific, lytic capacity of HSV1716.

5   The heterologous nitroreductase polypeptide expressed by
an herpes simplex virus according to the present
invention may be useful in gene directed enzyme-prodrug
targeting techniques for tissue specific delivery of
active pharmaceutical agents derived by nitroreductase
dependent activation of the NTR prodrug.

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In vivo, the inventors have demonstrated that the
nitroreductase gene, when introduced by HSV1716/CMV-
NTR/GFP into mouse gliomal xenograft models in
combination with the prodrug CB1954, results in delay in

15   tumour growth and in oncolysis. Administering both the
HSV1716/CMV-NTR/GFP virus and CB1954 prodrug in
combination was observed to produce a greater effect than
either virus or prodrug alone, i.e. the combination
exhibits a synergistic effect.

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The results demonstrate that the combination of oncolytic
HSV therapy with gene therapy directed
nitroreductase/prodrug treatment provides an effective
means of tumour cell kill and tumour growth reduction and

25   thereby a treatment for tumour.

Combining herpes simplex virus HSV1716-mediated oncolysis
with nitroreductase gene transfer has yielded results
exhibiting a surprising synergy and provides a novel

30   therapeutic strategy for treatment of tumours of all
kinds.

HSV1716/CMV-NTR/GFP has been deposited in the name of
Crusade Laboratories Limited having an address at
Department of Neurology Southern General Hospital 1345
Govan Road Govan Glasgow G51 5TF Scotland on 05 November
5   2003 at the European Collection of Cell Cultures (ECACC),
Health Protection Agency, Porton Down, Salisbury,
Wiltshire, SP4 0JG, United Kingdom under accession number
03110501 in accordance with the provisions of the
Budapest Treaty on the International Recognition of the
10   Deposit of Microorganisms for the Purposes of Patent
Procedure (herein referred to as the 'Budapest Treaty').

Accordingly, the present invention relates to a herpes
simplex virus, wherein the herpes simplex virus genome
15   comprises a nucleic acid sequence encoding a
nitroreductase. The herpes simplex virus may also be
non-neurovirulent.

At its most general the present invention relates to an
20   herpes simplex virus wherein the herpes simplex virus
genome comprises nucleic acid encoding a nitroreductase.

According to one aspect of the present invention there is
provided an herpes simplex virus wherein the herpes
25   simplex virus genome comprises nucleic acid encoding an
heterologous nitroreductase (NTR).

Said nucleic acid may encode an E.coli NTR and may
comprise, consist of or include the nucleic acid sequence
30   of SEQ ID No. 2. Alternatively the nucleic acid may have
at least 60% sequence identity to SEQ ID No. 2. Said
degree of sequence identity may alternatively be one of

at least 70%, 80%, 90%, 95%, 96%, 97%, 98% or 99%
provided the polypeptide or protein encoded by such
nucleic acid has a nitroreductase function. Identity of
sequences is determined across the entire length of a

5   given nucleotide sequence. Where sequences are of
different length, sequence identity of the shorter
sequence is determined over the entire length of the
longer sequence.

10   Said nucleic acid encoding NTR may be selected by its
ability to hybridise to the nucleic acid of SEQ ID No. 2,
or its complement, under high stringency conditions.

The genome of said herpes simplex virus may further
15   comprise a regulatory sequence operably linked to said
nucleic acid encoding NTR, wherein said regulatory
sequence has a role in controlling transcription of said
nucleic acid.

20   The nucleic acid encoding NTR may be located in at least
one RL1 locus of the herpes simplex virus genome.
Suitably it may be located in, or overlap, at least one
of the ICP34.5 protein coding sequences of the herpes
simplex virus genome. The nucleic acid may be located in
25   both (this will usually be all) copies of the RL1 locus
or ICP34.5 protein coding sequence.

The herpes simplex virus is preferably a mutant and may
be a mutant of HSV-1 or HSV-2, more preferably of one of
30   HSV-1 strains 17, F or HSV-2 strain HG52. The herpes
simplex virus may be a further mutant of HSV-1 strain 17
mutant 1716.

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In certain arrangements the herpes simplex virus may be a
gene specific null mutant, such as an ICP34.5 null
mutant.

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In other arrangements the herpes simplex virus may lack
at least one expressible ICP34.5 gene.

In yet another arrangement the herpes simplex virus may
10   lack only one expressible ICP34.5 gene.

In yet another arrangement the herpes simplex virus may
be non-neurovirulent.

15   In herpes simplex viruses of the present invention the
nucleic acid encoding the NTR may form part of a nucleic
acid cassette permanently integrated in the herpes
simplex virus genome, said cassette comprising nucleic
acid encoding :
20   (a)   said nucleic acid encoding NTR; and
                       nucleic acid encoding:
                (a)   a ribosome binding site; and
                (b)   a marker,
        wherein the nucleic acid encoding NTR is arranged
25   upstream (5´) of the ribosome binding site and the
        ribosome binding site is arranged upstream (5´) of the
        marker, wherein said ribosome binding site has a role in
        controlling transcription of said marker.

30   A regulatory nucleotide sequence may be located upstream
        (5′) of the nucleic acid encoding NTR, wherein the
        regulatory nucleotide sequence has a role in controlling

and regulating transcription of the nucleic acid encoding the NTR and hence expression of the resulting transcript and polypeptide. The regulatory sequence may comprise selected promoter or enhancer elements known to the

5   person skilled in the art, e.g. the CytoMegalovirus (CMV) promoter.   Suitably the regulatory sequence may be constitutive or inducible.

The components of the cassette are preferably arranged in

10   a predetermined order.

In one preferred arrangement, the nucleic acid encoding NTR is arranged upstream (i.e. 5') of the ribosome binding site and the ribosome binding site is arranged

15   upstream (i.e. 5') of the marker. During transcription a single transcript may be produced from the cassette comprising a first cistron comprising nucleic acid encoding NTR (e.g. an mRNA transcript) and a second cistron comprising nucleic acid encoding the marker

20   wherein the ribosome binding site is located between the cistrons.

A transcription product of this cassette may be a bi- or poly- cistronic transcript comprising a first cistron

25   encoded by the nucleic acid encoding NTR and a second cistron encoding the marker nucleic acid wherein the ribosome binding site is located between said first and second cistrons.

30   In another arrangement, the nucleic acid encoding the NTR may be arranged upstream (i.e. 5') of a first regulatory

nucleotide sequence and the first regulatory nucleotide
sequence is arranged upstream (i.e. 5') of the marker.

The cassette may disrupt a protein coding sequence of the
herpes simplex virus genome resulting in inactivation of
the respective gene product.

One suitable ribosome binding site comprises a ribosome
entry site permitting entry of a ribosome to the
transcribed mRNA encoded by the nucleic acid of the
cassette such that the ribosome binds to the translation
start signal. Preferably, the ribosome entry site is an
internal ribosome entry site (IRES), more preferably an
encephalomyocarditis virus IRES, permitting cap-
independent initiation of translation. The IRES thus
enables translation of a coding sequence located
internally of a bi- or poly- cistronic mRNA, i.e. of a
cistron located downstream of an adjacent cistron on a
single transcript.

Preferably the marker is a defined nucleotide sequence
coding for a polypeptide which can be expressed in a cell
line (e.g. BHK cells) infected with mutant herpes simplex
virus into which the cassette has been recombined. The
function of the marker is to enable identification of
virus plaques containing mutant virus transformed with
the cassette.

The marker is preferably a detectable marker, more
preferably an expressible marker polypeptide or protein
comprising at least the coding sequence for the selected
polypeptide or protein. The nucleic acid encoding the

marker may further comprise regulatory sequence upstream
and/or downstream of the coding sequence having a role in
control of transcription of the marker mRNA. Preferred
markers include the Green Fluorescent Protein (GFP)
5   protein coding sequence or gene, preferably the enhanced
Green Fluorescent Protein (EGFP) protein coding sequence
or gene.

In other arrangements the marker may comprise a defined
10   nucleotide sequence which can be detected by
hybridisation under high stringency conditions with a
corresponding labelled nucleic acid probe, e.g. using a
fluorescent- or radio-label.

15   The cassette may also comprise nucleic acid encoding a
polyadenylation ("polyA") sequence, which sequence is
preferably located downstream (3´) of the nucleic acid
encoding the marker. One preferred polyA sequence is the
Simian Virus 40 (SV40) polyadenylation sequence. The
20   preferred location of the polyA sequence within the
cassette is immediately downstream (i.e. 3´) of the
marker.

Mutant herpes simplex viruses of the present invention
25   may be generated by site directed insertion of a nucleic
acid cassette into the viral genome, more preferably by
homologous recombination. However, the viruses of the
invention are not limited to Herpes simplex viruses
obtained in this way.
30

In other aspects of the present invention herpes simplex
viruses according to the present invention are provided

for use in a method of medical treatment. Suitably they are provided for use in the treatment of disease. Preferably they are provided for use in the treatment of cancer. Suitably they may be provided for use in the

5   oncolytic treatment of cancer/a tumour. The use of herpes simplex viruses according to the present invention in the manufacture of a medicament for the treatment of cancer is also provided.

10 In another aspect of the present invention medicaments comprising herpes simplex virus mutants according to the present invention for use in oncotherapy and methods of treating tumours comprising administering to a patient in need of treatment an effective amount of a mutant HSV or

15 a medicament comprising or derived from such HSV are also provided. Methods of lysing or killing tumour cells in vitro or in vivo comprising the step of administering to a patient in need of treatment an amount of an Herpes simplex virus according to the present invention are also

20 provided.

A medicament, pharmaceutical composition or vaccine comprising an Herpes simplex virus according to the present invention is also provided. The medicament,

25 pharmaceutical composition or vaccine may further comprise a pharmaceutically acceptable carrier, adjuvant or diluent. Pharmaceutical compositions or vaccines may further comprise an NTR prodrug.

30 The present invention may also include the following aspects which may be provided in combination with any of the other aspects and features described.

According to another aspect of the present invention a
herpes simplex virus is provided, wherein the genome of
said virus comprises a nucleic acid sequence encoding an

5   heterologous nitroreductase (NTR) in at least one of the
long repeat regions (R_L).

According to another aspect of the present invention a
herpes simplex virus is provided, wherein the genome of

10   said virus comprises a nucleic acid sequence encoding an
heterologous nitroreductase (NTR) and wherein the herpes
simplex virus is non-neurovirulent.

A composition comprising a herpes simplex virus of the

15   invention may be provided in combination with an NTR
prodrug. The NTR prodrug may be CB1954.

According to another aspect of the present invention a
herpes simplex virus for use in the treatment of a tumour

20   is provided, wherein the genome of said virus comprises a
nucleic acid sequence encoding an heterologous
nitroreductase in at least one of the long repeat regions
(R_L).

25   According to another aspect of the present invention a
herpes simplex virus for use in the treatment of a tumour
is provided, wherein the genome of said virus comprises a
nucleic acid sequence encoding an heterologous
nitroreductase and wherein the herpes simplex virus is

30   non-neurovirulent.

According to another aspect of the present invention a
herpes simplex virus is provided, wherein the genome of
said virus comprises a nucleic acid sequence encoding an
heterologous nitroreductase in at least one of the long
5   repeat regions (R_L), for use, in combination with an NTR
prodrug, in the treatment of a tumour.

According to another aspect of the present invention a
herpes simplex virus is provided, wherein the genome of
10   said virus comprises a nucleic acid sequence encoding an
heterologous nitroreductase and wherein the herpes
simplex virus is non-neurovirulent, for use, in
combination with an NTR prodrug, in the treatment of a
tumour.
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According to another aspect of the present invention a
kit of parts is provided comprising a first container
having a quantity of an herpes simplex virus of the
invention and a second container having a quantity of an
20   NTR prodrug.

In another aspect the use of a herpes simplex virus,
wherein the genome of said virus comprises a nucleic acid
sequence encoding an heterologous nitroreductase in at
25   least one of the long repeat regions (R_L), in the
manufacture of a medicament for the treatment of a tumour
is also provided.

In another aspect the use of a herpes simplex virus,
30   wherein the genome of said virus comprises a nucleic acid
sequence encoding an heterologous nitroreductase and
wherein the herpes simplex virus is non-neurovirulent, in

the manufacture of a medicament for the treatment of a
tumour is also provided.

In another aspect the use in the manufacture of a
5   medicament for the treatment of a tumour of a herpes
simplex virus, wherein the genome of said virus comprises
a nucleic acid sequence encoding an heterologous
nitroreductase in at least one of the long repeat regions
(R_L), and an NTR prodrug is also provided.
10

In another aspect the use in the manufacture of a
medicament for the treatment of a tumour of a herpes
simplex virus, wherein the genome of said virus comprises
a nucleic acid sequence encoding an heterologous
15   nitroreductase and wherein the herpes simplex virus is
non-neurovirulent, and an NTR prodrug is also provided.

In another aspect the use of a herpes simplex virus,
wherein the genome of said virus comprises a nucleic acid
20   sequence encoding an heterologous nitroreductase in at
least one of the long repeat regions (R_L) in the
manufacture of a first medicament for administering
sequentially or simultaneously with a second medicament
comprising an NTR prodrug in the treatment of a tumour is
25   also provided

In another aspect the use of an NTR prodrug in the
manufacture of a first medicament for administering
sequentially or simultaneously with a second medicament
30   comprising a herpes simplex virus, wherein the genome of
said virus comprises a nucleic acid sequence encoding an
heterologous nitroreductase in at least one of the long

repeat regions (R_L), in the treatment of a tumour is also provided.

In another aspect the use of an NTR prodrug in the
5   manufacture of a first medicament for administering
sequentially or simultaneously with a second medicament
comprising a herpes simplex virus, wherein the genome of
said virus comprises a nucleic acid sequence encoding an
heterologous nitroreductase and wherein the herpes
10   simplex virus is non-neurovirulent, in the treatment of a
tumour is also provided.

In another aspect the use of a herpes simplex virus,
wherein the genome of said virus comprises a nucleic acid
15   sequence encoding an heterologous nitroreductase and
wherein the herpes simplex virus is non-neurovirulent, in
the manufacture of a first medicament for administering
sequentially or simultaneously with a second medicament
comprising an NTR prodrug, in the treatment of a tumour
20   is also provided.

The time period between sequential administrations may be
such that the herpes simplex virus and NTR prodrug may
interact in the body to produce an active pharmaceutical
25   agent in situ. Preferred time periods may be less than 15
minutes, less than one hour, two hours, three hours, four
hours, five hours or six hours, twelve hours, twenty four
hours, forty eight hours, one week or two weeks. Either
the herpes simplex virus or NTR prodrug may be
30   administered first.

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In another aspect a method of treatment of a tumour is
provided comprising the steps of:

    (i)   administering to a patient in need of treatment
             a herpes simplex virus, wherein the genome of

5   said virus comprises a nucleic acid sequence
             encoding a nitroreductase in at least one of
             the long repeat regions (R_L); and

    (ii)   administering to said patient a therapeutically
             effective amount of an NTR prodrug.

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In another aspect a method of treatment of a tumour is
provided comprising the steps of:

    (i)   administering to a patient in need of treatment
             a herpes simplex virus, wherein the genome of

15   said virus comprises a nucleic acid sequence
             encoding a nitroreductase and wherein the
             herpes simplex virus is non-neurovirulent; and

    (ii)   administering to said patient a therapeutically
             effective amount of an NTR prodrug.

20

In the methods of treatment said herpes simplex virus is
preferably capable of killing tumour cells, e.g. by
oncolysis.

25 In aspects of the invention involving an NTR prodrug, one
preferred prodrug is CB1954.

In another aspect a method of expressing in vitro or in
vivo a nitroreductase is provided, said method comprising

30 the step of infecting at least one cell or tissue of
interest with a herpes simplex virus, wherein the genome
of said virus comprises a nucleic acid sequence encoding

a heterologous nitroreductase in at least one of the long
repeat regions (R_L), said nitroreductase operably linked
to a transcription regulatory sequence.

5   In another aspect a method of expressing in vitro or in
vivo a nitroreductase is provided, said method comprising
the step of infecting at least one cell or tissue of
interest with a non-neurovirulent herpes simplex virus,
wherein the genome of said virus comprises a nucleic acid
10   sequence encoding a heterologous nitroreductase, said
nitroreductase operably linked to a transcription
regulatory sequence.

Herpes simplex viruses of the invention having nucleic
15   acid encoding an heterologous nitroreductase in at least
one of the long repeat regions (R_L) of the HSV genome
preferably have said nucleic acid in each of the long
repeat regions of the HSV genome. Two long repeat regions
are usually present in the HSV genome.
20
The NTR nucleotide sequence may encode a full length
transcript or polypeptide (i.e. comprise the complete NTR
protein coding sequence). Alternatively, provided the
polypeptide product retains nitroreductase activity, the
25   NTR nucleotide sequence may comprise one or more
fragments of the full length sequence respectively coding
for a fragment of the full length transcript or a
truncated polypeptide.

30   A fragment may comprise a nucleotide sequence encoding at
least 10% of the corresponding full length sequence, more
preferably the fragment comprises at least 20, 30, 40,

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50, 60, 70, 80, 85, 90, 95, 96, 97, 98 or 99% of the
corresponding full length sequence. Preferably, the
fragment comprises at least, i.e. has a minimum length
of, 20 nucleotides, more preferably at least 30, 40, 50,

5   100, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000,
1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900
2000, 2100, 2200, 2300, 2400, 2500, 2600, 2700, 2800,
2900, 3000, 3100, 3200, 3300, 3400, 3500, 3600, 3700,
3800, 3900 or 4000 nucleotides. The fragment may have a

10   maximum length, i.e. be no longer than, 20 nucleotides,
more preferably no longer than 30, 40, 50, 100, 150, 200,
300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200,
1300, 1400, 1500, 1600, 1700, 1800, 1900 2000, 2100,
2200, 2300, 2400, 2500, 2600, 2700, 2800, 2900, 3000,

15   3100, 3200, 3300, 3400, 3500, 3600, 3700, 3800, 3900 or
4000. The fragment length may be anywhere between said
minimum and maximum length.

In one preferred arrangement, the mutant HSV is

20   HSV1716/CMV-NTR/GFP deposited in the name of Crusade
Laboratories Limited having an address at Department of
Neurology Southern General Hospital 1345 Govan Road Govan
Glasgow G51 5TF Scotland on 05 November 2003 at the
European Collection of Cell Cultures (ECACC), Health

25   Protection Agency, Porton Down, Salisbury, Wiltshire, SP4
0JG, United Kingdom under accession number 03110501 in
accordance with the provisions of the Budapest Treaty.

Suitably, the administration of said herpes simplex virus

30   and/or said NTR prodrug may comprise parenteral
administration. Preferably administration of the herpes
simplex virus is by injection, more preferably injection

to the tumour which is to be treated.   The NTR prodrug
may also be administered by injection, which may also
comprise direct injection to the site of the tumour.
Alternatively injections may be intravenous.

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Administration of the herpes simplex virus and NTR
prodrug may be simultaneous, e.g. by combining virus and
prodrug in a single composition, or be substantially
simultaneous, e.g. one being administered immediately

10   after the other.   Alternatively, a predetermined time
period may be provided between administration of the
herpes simplex virus and the NTR prodrug.   The invention
is not limited by the order of administration.

15   In a further aspect of the present invention in vitro or
in vivo methods are provided for delivery of nucleic acid
encoding a nitroreductase to at least one cell or to a
tissue of interest said method comprising the step of
infecting said cell(s) or tissue with a herpes simplex

20   virus according to the invention.

In another aspect of the invention, a kit of parts is
provided comprising a first container in which a quantity
of herpes simplex virus according to the invention is

25   provided and a second container in which a quantity of
NTR prodrug is provided.   Instructions for the
administration, optionally including information on
suitable dosages of herpes simplex virus and/or the NTR
prodrug, may also be provided with the kit.

30

In another aspect of the present invention a method of
making or producing a modified herpes simplex virus of

the invention is provided comprising the step of
introducing a nucleic acid sequence encoding a
nitroreductase at a selected and/or predetermined
insertion site in the genome of a selected herpes simplex

5   virus.

As described, the nucleic acid sequence encoding the
nitroreductase may form part of a nucleic acid cassette
which is inserted in the genome of a selected herpes

10   simplex virus by homologous recombination. Whether part
of a cassette or not, the site of insertion may be in any
genomic location selected. One preferred insertion site
is in one or both of the long repeat regions (R_L), and one
copy of the cassette is preferably inserted in each copy

15   of the long repeat (R_L). More preferably the insertion
site is in at least one (preferably both) RL1 locus and
most preferably it is inserted in at least one
(preferably both) of the ICP34.5 protein coding sequences
of the HSV genomic DNA. It is preferred that the

20   insertion occurs in identical or substantially similar
positions in each of the two repeat regions, RL1 loci or
ICP34.5 protein coding sequences.

Insertion may be such as to produce a modified virus

25   which is a non-neurovirulent mutant capable of expressing
the encoded nitroreductase polypeptide upon transfection
into mammalian, more preferably human, cells in vivo and
in vitro in a form which is functional to facilitate the
uptake and/or activation of NTR prodrug. The non-

30   neurovirulent mutant may be an ICP34.5 null mutant.

The nucleic acid cassette may be of any size, e.g. up to 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50kbp in length.

Preferably, the herpes simplex virus contains at least
5   one copy of the nucleic acid encoding the nitroreductase in each long repeat region (R_L), i.e. in the terminal and internal long repeat (TR_L and IR_L) regions. In a preferred arrangement each exogenous sequence or cassette is located in an RL1 locus of the herpes simplex virus
10   genome, more preferably in the DNA of the herpes simplex virus genome encoding the ICP34.5 gene or protein coding sequence. The herpes simplex virus thereby lacks neurovirulence.

15   The parent herpes simplex virus, from which a virus of the invention is derived may be of any kind, e.g. HSV-1 or HSV-2. In one preferred arrangement the herpes simplex virus is a variant of HSV-1 strain 17 and may be obtained by modification of the strain 17 genomic DNA.
20   Suitable modifications include the insertion of the exogenous nitroreductase nucleic acid sequence or exogenous/ heterologous cassette comprising said sequence into the herpes simplex virus genomic DNA. The insertion may be performed by homologous recombination of the
25   exogenous nucleic acid sequence into the genome of the selected herpes simplex virus.

Although the non-neurovirulent phenotype of the herpes simplex virus of the invention may be the result of
30   insertion of the exogenous nucleic acid sequence in the RL1 locus, herpes simplex viruses according to the present invention may be obtained by utilising a non-

neurovirulent parent strain, e.g. HSV1716 deposited under
the Budapest Treaty at the European Collection of Animal
Cell Cultures (ECACC), Health Protection Agency, Porton
Down, Salisbury, Wiltshire, United Kingdom under
5   accession number V92012803, and inserting the exogenous
nucleic acid sequence at another location of the genome
by standard genetic engineering techniques, e.g.
homologous recombination. In this aspect the location of
the herpes simplex virus genome selected for insertion of
10   the nitroreductase nucleic acid sequence or cassette
containing said sequence may be a neutral location.

Herpes simplex viruses of the present invention may be
variants of a known 'parent' strain from which the herpes
15   simplex virus of the invention has been derived. A
particularly preferred parent strain is HSV-1 strain 17.
Other parent strains may include HSV-1 strain F or HSV-2
strain HG52. A variant comprises an HSV in which the
genome substantially resembles that of the parent,
20   contains the nitroreductase nucleic acid sequence or
cassette containing said sequence and may contain a
limited number of other modifications, e.g. one, two or
three other specific mutations, which may be introduced
to disable the pathogenic properties of the herpes
25   simplex virus, for example a mutation in the
ribonucleotide reductase (RR) gene, the 65K trans
inducing factor (TIF) and/or a small number of mutations
resulting from natural variation, which may be
incorporated naturally during replication and selection
30   in vitro or in vivo. Otherwise the genome of the variant
will be that of the parent strain.

Herpes simplex viruses of the invention may be used
alone, or in combination with an NTR prodrug in a method
of medical treatment. This may involve treatment of
diseases associated with or involving the proliferation
5   of cells, or cancers or tumours of any kind.   Treatment
may involve the selective lysis of dividing cells. This
may be oncolysis, i.e. lysis of tumour cells. Tumours to
be treated may be of any kind, may comprise cancers,
neoplasms or neoplastic tissue and may be in any animal
10   or human patient.

Cancer/tumour types which may be treated may be primary
or secondary (metastatic) tumours. Tumours to be treated
may be nervous system tumours originating in the central
15   or peripheral nervous system, e.g. glioma,
medulloblastoma, meningioma, neurofibroma, ependymoma,
Schwannoma, neurofibrosarcoma, astrocytoma and
oligodendroglioma, or may be non-nervous system.tumours
originating in non-nervous system tissue e.g. melanoma,
20   mesothelioma, lymphoma, hepatoma, epidermoid carcinoma,
prostate carcinoma, breast cancer cells, lung cancer
cells or colon cancer cells. HSV mutants of the present
invention may be used to treat metastatic tumours of the
central or peripheral nervous system which originated in
25   a non-nervous system tissue.

Herpes simplex viruses of the invention may be used in
'gene delivery' methods in vitro or in vivo. Non-
neurovirulent herpes simplex viruses of the invention are
30   expression vectors and may be used to infect selected
cells or tissues in order to express the nitroreductase
encoded by the herpes simplex virus genome.

In one arrangement, cells may be taken from a patient, a
donor or from any other source, infected with a herpes
simplex virus of the invention, optionally screened for
5   expression and/or function of the encoded nitroreductase,
and optionally returned/introduced to a patient's body,
e.g. by injection.

Delivery of herpes simplex viruses of the invention to
10   the selected cells may be performed using naked virus or
by encapsulation of the virus in a carrier, e.g.
nanoparticles, liposomes or other vesicles.

In vitro cultured cells, preferably human or mammalian
15   cells, transformed with viruses of the present invention
and preferably cells expressing the nitroreductase
protein as well as methods of transforming such cells in
vitro with said viruses form further aspects of the
present invention.
20

In this specification, a mutant herpes simplex virus is a
non-wild type herpes simplex virus and may be a
recombinant herpes simplex virus. Mutant herpes simplex
viruses may comprise a genome containing modifications
25   relative to the wild type. A modification may include at
least one deletion, insertion, addition or substitution.

Medicaments and pharmaceutical compositions according to
aspects of the present invention may be formulated for
30   administration by a number of routes, including but not
limited to, parenteral, intravenous, intramuscular,
intratumoural, oral and nasal. The medicaments and

compositions may be formulated in fluid or solid (e.g. tablet) form. Fluid formulations may be formulated for administration by injection to a selected region of the human or animal body.

5

In this specification, non-neurovirulence is defined by the ability to introduce a high titre of virus (approx 10^6 plaque forming units (pfu)) to an animal or patient[22, 23] without causing a lethal encephalitis such that the LD_{50}

10   in animals, e.g. mice, or human patients is in the approximate range of $\geq 10^6$ pfu[21].

Where all copies of the ICP34.5 gene present in the herpes simplex virus genome (two copies are normally

15   present) are disrupted such that the herpes simplex virus is incapable of producing a functional ICP34.5 gene product, the virus is considered to be an ICP34.5 null mutant.

20   A regulatory sequence (e.g. promoter) that is operably linked to a nucleotide sequence may be located adjacent to that sequence or in close proximity such that the regulatory sequence can effect and/or control expression of a product of the nucleotide sequence. The encoded

25   product of the nucleotide sequence may therefore be expressible from that regulatory sequence.

NTR prodrug

30   In this specification, "NTR prodrug" means any chemical compound or agent which is not toxic, or exhibits low toxicity, to a selected human or animal body, or to

selected cells or tissues of the human or animal body, and which may be activated by a nitroreducase enzyme to a chemical compound or agent which is cytotoxic to the human or animal body or to those selected cells.

5

"Activation" may involve conversion of a non-toxic (or low toxicity) prodrug to an active cytotoxic form. That conversion may involve enzymatic reduction of the prodrug by NTR. The enzymatic reduction reaction may involve the

10 prodrug as a substrate for NTR and may involve other co-factors.

Examples of NTR prodrugs may include compounds from the following classes of molecules:

15

    1.   dinitirobenzamides;

    2.   dinitroaziridinylbenzamides (e.g. CB1954);

    3.   dinitrobenzamide mustard derivatives (e.g. SN23862);

20   4.   4-nitrobenzylcarbamates;

    5.   nitroindolines;

    6.   nitroaromatics that are substrates of NTR and are activated to release a cytotoxic phosphoramide mustard or like-reactive species upon NTR-

25   reduction (also called nitroaryl phosphoramides)[17];

    7.   nitroaromatic prodrugs of the dinitrobenzamide class.

Examples of NTR prodrugs are disclosed in references 16
30 and 17 which are incorporated herein in their entirety by reference.

28

Nitroreductase (NTR)

Nitroreductase enzymes commonly catalyze the reduction of
nitro compounds, quinones, and dyes. The enzymatic
5   reduction may involve the co-factor NADPH.

In this specification nitroreductase (NTR) refers to an
enzyme capable of activating an NTR prodrug to an active
cytotoxic form.

10

Preferred NTR's may have the ability to reduce a wide
range of nitro-containing compounds such as nitrofurazone
(to the hydroxylamines) and quinones such as menadione
(to the quinols).

15

Preferred NTR's may be specifically inhibited by the
irreversible inhibitor dicoumarol.

One preferred NTR is the E.coli oxygen insensitive
20   nitroreductase enzyme (the nfsB gene product). Sequence
information for E.coli NTR can be found at the NCBI
database (http://www.ncbi.nlm.nih.gov/) under accession
numbers BA000007 (GI:47118301) - E.coli complete genome
sequence - and BAB34039 (GI:13360074) - nitroreductase
25   sequence information.

The amino acid sequence for the E.coli NTR protein (SEQ
ID No. 1) and polynucleotide sequence for the E.coli NTR
gene (SEQ ID No. 2) are reproduced at Figure 32 (A) and
30   (B) respectively.

The nucleotide and amino acid sequences of suitable
nitroreductase enzymes may be derived or obtained from
any animal, insect or microorganism including humans,
non-human mammals and bacteria and may be selected from
those sequences which are publicly available. Many
sequences for other nitroreductase genes are publicly
available. Examples of other nitroreductase nucleic acid
sequences which may form part of a herpes simplex virus
according to the present invention include the following
which are referred to by their accession number for the
NCBI database (www.ncbi.nlm.nih.gov):

- BAB34039   (GI:13360074) - E.coli
- BAA35218.1   (GI:1651240) - E.coli
- AAB72053.1   (GI:2415385) - B.subtilis.

Hybridisation stringency

In accordance with the present invention, nucleic acid
sequences may be identified by using hybridization and
washing conditions of appropriate stringency.

Complementary nucleic acid sequences will hybridise to
one another through Watson-Crick binding interactions.
Sequences which are not 100% complementary may also
hybridise but the strength of the hybridisation usually
decreases with the decrease in complementarity. The
strength of hybridisation can therefore be used to
distinguish the degree of complementarity of sequences
capable of binding to each other.

The "stringency" of a hybridization reaction can be
readily determined by a person skilled in the art.

The stringency of a given reaction may depend upon
factors such as probe length, washing temperature, and
salt concentration. Higher temperatures are generally
5   required for proper annealing of long probes, while
shorter probes may be annealed at lower temperatures. The
higher the degree of desired complementarity between the
probe and hybridisable sequence, the higher the relative
temperature which can be used. As a result, it follows
10   that higher relative temperatures would tend to make the
reaction conditions more stringent, while lower
temperatures less so.

For example, hybridizations may be performed, according
15   to the method of Sambrook et al., ("Molecular Cloning, A
Laboratory Manual, Cold Spring Harbor Laboratory Press,
1989) using a hybridization solution comprising: 5X SSC,
5X Denhardt's reagent, 0.5-1.0% SDS, 100 µg/ml denatured,
fragmented salmon sperm DNA, 0.05% sodium pyrophosphate
20   and up to 50% formamide. Hybridization is carried out at
37-42°C for at least six hours. Following hybridization,
filters are washed as follows: (1) 5 minutes at room
temperature in 2X SSC and 1% SDS; (2) 15 minutes at room
temperature in 2X SSC and 0.1% SDS; (3) 30 minutes-1
25   hour at 37°C in 1X SSC and 1% SDS; (4) 2 hours at 42-
65°C in 1X SSC and 1% SDS, changing the solution every 30
minutes.

One common formula for calculating the stringency
30   conditions required to achieve hybridization between
nucleic acid molecules is to calculate the melting
temperature T_m (Sambrook et al., 1989):

$$T_m = 81.5°C + 16.6Log [Na+] + 0.41(\% \ G+C) - 0.63 \ (\%$$
$$formamide) - 600/n$$

5   where n is the number of bases in the oligonucleotide.

As an illustration of the above formula, using [Na+] =
[0.368] and 50% formamide, with GC content of 42% and an
average probe size of 200 bases, the T_m is 57°C. The T_m

10   of a DNA duplex decreases by 1 - 1.5°C with every 1%
decrease in sequence complementarity.

Accordingly, nucleotide sequences can be categorised by
an ability to hybridise to a target sequence under

15   different hybridisation and washing stringency conditions
which can be selected by using the above equation. The T_m
may be used to provide an indicator of the strength of
the hybridisation.

20   The concept of distinguishing sequences based on the
stringency of the conditions is well understood by the
person skilled in the art and may be readily applied.

Sequences exhibiting 95-100% sequence complementarity may

25   be considered to hybridise under very high stringency
conditions, sequences exhibiting 85-95% complementarity
may be considered to hybridise under high stringency
conditions, sequences exhibiting 70-85% complementarity
may be considered to hybridise under intermediate

30   stringency conditions, sequences exhibiting 60-70%
complementarity may be considered to hybridise under low
stringency conditions and sequences exhibiting 50-60%%

complementarity may be considered to hybridise under very
low stringency conditions.

The invention includes the combination of the aspects and
5   preferred features described except where such a
combination is clearly impermissible or expressly
avoided.

Aspects and embodiments of the present invention will now
10   be illustrated, by way of example, with reference to the
accompanying figures. Further aspects and embodiments
will be apparent to those skilled in the art. All
documents mentioned in this text are incorporated herein
by reference.
15

Brief Description of the Figures

Figure 1. Generation of plasmid RL1.dIRES-GFP from
plasmids pNAT-IRES-GFP and RL1.del.
20

Figure 2. Agarose gel electrophoresis of HpaI digested,
CIP treated, RL1.del. RL1.del was digested with HpaI. The
digested DNA was then treated with Calf Intestinal
Phosphatase (CIP) to prevent the vector re-annealing to
25   itself in subsequent ligation reactions. A sample of the
digested/CIP treated DNA was electrophoresed, beside a
1Kbp DNA ladder (Promega), on a 1% agarose gel. HpaI
linearises the vector at 8.6 Kbp.

30   Figure 3. Agarose gel electrophoresis of NsiI/SspI
digested pNAT-IRES-GFP (A) and purified/blunt-ended pCMV-
NAT-IRES-GFP-PolyA (B). Four NsiI/SspI digestions of

pNAT-IRES-GFP were electrophoresed, beside a 1Kbp DNA
Ladder (Promega) on a 1% agarose gel. The 5.4Kbp
fragments (pCMV-NAT-IRES-GFP-PolyA) were purified from
the gel. The purified DNA was blunt ended using Klenow
5   polymerase and a sample electrophoresed on an agarose gel
to check its concentration.

Figure 4. Identification of RL1.del clones containing the
pCMV-NAT-IRES-GFP-PolyA insert. Ligation reactions were
10   set up with the purified, blunt ended pCMV-NAT-IRES-GFP-
PolyA fragment and *Hpa*I digested, CIP treated RL1.del.
Bacteria were transformed with samples from the ligation
reactions and plated out onto LBA (Amp^r) plates. Colonies
were picked and plasmid DNA was extracted and digested
15   with *Afl*II. Digested samples were electrophoresed, beside
a 1Kbp DNA ladder (L) (Promega), on a 1% agarose gel.
*Clones 5 and 8 contained the pCMV-NAT-IRES-GFP-PolyA
insert as two fragments of the predicted size - 4.8Kbp
and 9.2Kbp - were generated from *Afl*II digestion. Clones
20   without inserts would not be digested with *Afl*II as there
is no *Afl*II site in RL1.del.
N.B. Inserts could have been cloned in two orientations,
both of which were acceptable.

25   **Figure 5**. Determination of the orientation of pCMV-NAT-
IRES-GFP-PolyA in clone 5 (RL1.dCMV-NAT-GFPb). pCMV-NAT-
IRES-GFP-PolyA (blunt ended) could have been cloned into
the *Hpa*I site of RL1.del in two orientations. To
determine the orientation of the insert in clone 5, the
30   plasmid was digested with *Xho*I and the digested DNA
electrophoresed, beside a 1Kbp DNA ladder (Promega), on a
1% agarose gel. If the insert had been cloned in the

orientation shown in A, two fragments of 10.2Kbp and
3.8Kbp would be generated from XhoI digestion. If it had
been cloned in the opposite orientation (B), two
fragments of 12.4Kbp and 1.6Kbp would be generated. The
5   presence of two fragments of 10.2Kbp and 3.8Kbp in the
gel confirmed that the insert had been cloned in the
orientation shown in A.
*This XhoI site was present in the initial cloning vector
(RL1.del), upstream of the HpaI site into which pCMV-
10   NAT-IRES-GFP-PolyA was cloned.

Figure 6. Removal of pCMV-NAT from clone 5 (A) and large
scale plasmid preparation of RL1.dIRES-GFP (B). Four
samples of clone 5 were digested with XhoI and
15   electrophoresed, beside a 1Kbp DNA ladder (L) (Promega),
on a 1% agarose gel (A). The larger fragment of DNA
generated from this digestion (10.2Kbp) was purified from
the gel and ligated back together, at the XhoI sites, to
form a single XhoI site in a new plasmid, designated
20   RL1.dIRES-GFP. A large-scale plasmid preparation was
grown up and the preparation checked by digesting with
XhoI. 1µl and 4µl of the digested DNA was
electrophoresed, beside a 1Kbp DNA ladder (L) (Promega),
on a 1% agarose gel (B). The DNA should produce a single
25   fragment of 10.2Kbp when digested with XhoI. The ClaI,
BglII, NruI and XhoI sites of RL1.dIRES-GFP are all
unique.
*Clone 5 is the RL1.del plasmid into which has been
cloned the 5.4Kbp pCMV-NAT-IRES-GFP-PolyA fragment from
30   pNAT-IRES-GFP.

Figure 7. Generation, detection and purification of
ICP34.5 null HSV-1 expressing a gene product of interest.

Figure 8. Strategy used to clone pCMV-NTR from pPS949
5   into RL1.dIRES-GFP. (1) Digest pPS949 with *Bam*H1 and
purify the 1.6Kbp pCMV-NTR fragment; (2) Digest
RL1.dIRES-GFP with *Bgl*II and treat with Calf Intestinal
Phosphatase (CIP); (3) Clone the pCMV-NTR fragment (*Bam*HI
ends) into the *Bgl*II site of RL1.dIRES-GFP.
10   * The pPS949 plasmid was a kind gift from Professor
Lawrence Young (University of Birmingham) and contains
the *E.coli* nitroreductase (NTR) gene downstream of the
CMV-IE promoter (pCMV) in pLNCX (Clontech).

15   **Figure 9.** Agarose gel electrophoresis of *Bam*HI digested
pPS949 (A) and the purified pCMV-NTR fragment (B). Four
samples of pPS949 were digested with *Bam*HI and
electrophoresed, beside a 1Kbp DNA ladder (L) (New
England Biolabs), on a 1% agarose gel. The 1.6Kbp
20   fragments, consisting of the *E.coli* nitroreductase (NTR)
gene downstream of the CMV IE promoter (pCMV), were
purified from the gel and a sample of the purified DNA
was electrophoresed on an agarose gel to check its
concentration.
25

Figure 10. Agarose gel electrophoresis of *Bgl*II
digested, CIP treated RL1.dIRES-GFP.RL1.dIRES.GFP was
digested with *Bgl*II. The digested plasmid was then
treated with Calf Intestinal Phosphatase (CIP) to prevent
30   the vector re-annealing to itself in subsequent ligation
reactions. A sample of the digested/CIP treated DNA was
electrophoresed, beside a 1Kbp DNA ladder (Promega), on a

1% agarose gel to check its concentration. pCMV-NTR from
pPS949 was subsequently cloned into this digested/CIP
treated vector.

5   **Figure 11.** Determination of the orientation of pCMV-NTR
        in clone 4. pCMV-NTR (*Bam*HI ends) could have been cloned
        into the *Bgl*II site of RL1.dIRES-GFP in two orientations.
        To determine the orientation, clone 4 was digested with
        *Bgl*II and *Xho*I and the digested DNA electrophoresed,
10   beside a 1Kbp DNA ladder (Promega), on a 1% agarose gel.
        If the insert was in the desired orientation (A), two
        fragments (11.5Kbp and 300bp) would be generated. If in
        the opposite orientation, two fragments of 10.5Kbp and
        1.3Kbp would be generated. The presence of a band at
15   ~300bp (and the absence of a band at 1.3Kbp) confirmed
        that the pCMV-NTR fragment had been cloned into the
        vector in the desired orientation.

        Figure 12.   Agarose gel electrophoresis of *Sca*I digested
20   clone 4 (A) and HSV1716/CMV-NTR/GFP viral titres (B).
        Clone 4 (RL1.dCMV-NTR-GFP) was digested with *Sca*I, the
        digested DNA purified and 5μl electrophoresed, beside a
        1Kbp DNA ladder (Promega), on a 1% agarose gel, to check
        its concentration. 80% confluent BHK cells were then co-
25   transfected with 10μl HSV17^{+} DNA and an appropriate volume
        of the remaining digested clone 4. The cells were
        incubated at 37°C for 3 days until cpe was evident.
        Recombinant viral plaques were picked under the
        fluorescent microscope, purified and a virus stock, named
30   HSV1716/CMV-NTR/GFP, grown up. The cell-associated and
        cell-released fraction of the virus stock was titrated on
        BHK cells.

Figure 13. Growth kinetics of HSV17$^+$, HSV1716 and
HSV1716/CMV-NTR/GFP in confluent BHK and 3T6 cells.
Confluent BHK and 3T6 cells were infected at a MOI of
5   0.1pfu/cell. Infected cells were harvested at 0, 4, 24,
48 and 72hrs post infection, sonicated and progeny virus
titrated on BHK cell monolayers. All viruses replicated
with similar kinetics in BHK cells (A); HSV1716 and
HSV1716/CMV-NTR/GFP both failed to replicate efficiently
10   in confluent 3T6 cells (B).

Figure 14. Western blot analysis of ICP34.5 expression in
HSV17$^+$ and HSV1716/CMV-NTR/GFP infected BHK cells. BHK
cells were infected with HSV17$^+$ and HSV1716/CMV-NTR/GFP at
15   a MOI of 10pfu/cell. 16hrs post infection, the cells were
harvested and protein extracts analysed using 10% SDS-
PAGE in a Western blot using a polyclonal anti-ICP34.5
antibody. ICP34.5 was strongly expressed in HSV17$^+$
infected cells but was not expressed in HSV1716/CMV-
20   NTR/GFP infected cells.

Figure 15. Western blot analysis of NTR expression in
HSV1716/CMV-NTR/GFP infected cell lines. BHK, C8161, VM and
3T6 cells were infected with 10pfu/cell HSV1716/CMV-NTR/GFP,
25   HSV17$^+$ or mock infected. 16hrs post infection, the cells were
harvested and protein extracts analysed in a Western blot
using a polyclonal NTR-specific antibody. Significant NTR
expression was detected in all the HSV1716/CMV-NTR/GFP
infected cells. No NTR expression was detected in the mock
30   or HSV17$^+$ infected cells.

Figure 16. Effect of HSV1716/CMV-NTR/GFP and HSV1716-GFP
with or without CB1954 (50µM) on confluent 3T6 cells.
Confluent 3T6 cells in three wells of a 96-well plate
were mock infected, infected with 1 or 10pfu/cell

5   HSV1716/CMV-NTR/GFP or infected with 1pfu/cell of
HSV1716-GFP. 45 minutes later, infected cells were
overlaid with media containing 50µM CB1954 or with media
alone and incubated at 37°C. 24, 48, 72, 96, and 120hrs
later, % cell survival was determined relative to that of

10   mock infected cells without prodrug using CellTiter 96
Aqueous One Solution Cell Proliferation Assay (Promega).
Figures shown represent the mean of 3 values +/- standard
error of the mean.

15   **Figure 17.** Effect of HSV1716/CMV-NTR/GFP and HSV1716-GFP
with or without CB1954 (50µM) on confluent C8161 cells.
Confluent C8161 cells in three wells of a 96-well plate
were mock infected, infected with 1 or 10pfu/cell
HSV1716/CMV-NTR/GFP or infected with 1pfu/cell of

20   HSV1716-GFP. 45 minutes later, infected cells were
overlaid with media containing 50µM CB1954 or with media
alone and incubated at 37°C. 24, 48 and 72hrs later, %
cell survival was determined relative to that of mock
infected cells without prodrug using CellTiter 96 Aqueous

25   One Solution Cell Proliferation Assay (Promega). Figures
shown represent the mean of 3 values +/- standard error
of the mean.

Figure 18. Confluent 3T6 cells 72hrs post treatment with
30   10pfu/cell HSV1716/CMV-NTR/GFP (A), or 10pfu/cell
HSV1716/CMV-NTR/GFP with 50µM CB1954 (B). The extent of

cell death is significantly more pronounced in
HSV1716/CMV-NTR/GFP infected cells overlaid with media
containing 50µM CB1954 than in HSV1716/CMV-NTR/GFP
infected cells overlaid with normal media. The extent of
5   cell death following infection of these cells with
10pfu/cell HSV1716, with or without CB1954, is comparable
to that seen in A (data not shown). 50µM CB1954 alone has
no effect on these cells.

10   **Figure 19.** Confluent C8161 cells 72hrs post treatment
with 10pfu/cell HSV1716/CMV-NTR/GFP (A), or 10pfu/cell
HSV1716/CMV-NTR/GFP with 50µM CB1954 (B). The extent of
cell death is significantly more pronounced in
HSV1716/CMV-NTR/GFP infected cells overlaid with media
15   containing 50µM CB1954 than in HSV1716/CMV-NTR/GFP
infected cells overlaid with normal media. The extent of
cell death following infection of these cells with
10pfu/cell HSV1716, with or without CB1954, is comparable
to that seen in A (data not shown). 50µM CB1954 alone has
20   no effect on these cells.

Figure 20.   Weight change (as a guide to health) in
athymic nude mice with subcutaneous A2780 (xenograft)
tumours injected intratumourally with HSV 1790. Group
25   size = 3 mice per dose. A2780 xenografts at date of
intratumoural injection (Day 0) are between 0.5 - 1mm in
diameter. The xenografts have reached this size 12 days
after injection with 10 million A2780 cells
subcutaneously on the flank of female athymic nude mice.

30

Figure 21.   Change in tumour volume over time in athymic nude mice with A2780 xenografts after intratumoural injection of HSV 1790.

5   **Figure 22.**   Starting tumour sizes of mice.

Figure 23.   Alterations in weight after treatment with CMV-ntr, CB1954 or a combination of both.

10   **Figure 24.**   Change in tumour volume after treatment with CMV-ntr, CB1954 or a combination of both.

Figure 25.   Starting tumour volume of each treatment group (see Table 2).

15

Figure 26.   Weight (as a measurement of health) in athymic nude mice with A2780 xenograft treated with either HSV 1790, HSV 1716, CB 1954 or a combination of them.

20

Figure 27.   Change in tumour volume of xenografts treated with the prodrug CB1954.

Figure 28.   Changes in tumour volume in xenograft
25   treated with 10^5 PFU HSV 1790 and CB1954.

Figure 29.   Changes in tumour volume in xenografts treated with 10^6 PFU HSV 1790 and CB1954.

30   **Figure 30.**   Changes in tumour volume in xenografts treated with 10^5 PFU HSV 1716 and CB1954.

Figure 31.   Comparison of 10^5 PFU, 10^6 PFU HSV 1790 and
10^5 PFU HSV 1716.

Figure 32.   Sequence information for E.coli NTR.   (A)
5   Amino acid sequence of NTR polypeptide (SEQ ID No.1); (B)
polynucleotide sequence for NTR gene (SEQ ID No.2).

Figure 33.   Structure of two NTR prodrugs. (A) CB1954;
(B) SN23862.

10

Detailed Description of the Best Mode of the Invention

Specific details of the best mode contemplated by the
inventors for carrying out the invention are set forth
15   below, by way of example. It will be apparent to one
skilled in the art that the present invention may be
practiced without limitation to these specific details.

Vectors Useful for Generation of Herpes Simplex virus
20   Mutants

Mutant herpes simplex viruses of the invention may be
generated by use of nucleic acid vectors.

25   One such vector useful for generation of mutant herpes
simplex viruses according to the present invention is a
nucleic acid vector comprising, consisting or consisting
essentially of:
first and second nucleotide sequences corresponding to
30   nucleotide sequences flanking an insertion site in the
genome of a selected herpes simplex virus; and

a cassette located between said first and second
nucleotide sequences comprising nucleic acid encoding:
   a)   one or a plurality of insertion sites; and
   b)   a ribosome binding site; and
5 c)   a marker.

Another vector useful for generation of mutant herpes
simplex viruses according to the present invention is a
nucleic acid vector comprising, consisting or consisting
10 essentially of:
first and second nucleotide sequences corresponding to
nucleotide sequences flanking an insertion site in the
genome of a selected herpes simplex virus; and
a cassette located between said first and second
15 nucleotide sequences comprising nucleic acid encoding:
   a)   one or a plurality of insertion sites; and
   b)   a first regulatory nucleotide sequence; and
   c)   a marker.

20 The first and second nucleotide sequences may correspond
to nucleotide sequences flanking an insertion site formed
in, or comprising all or a part of, the ICP34.5 protein
coding sequence of the genome of a selected herpes
simplex virus.
25
The cassette may comprise a plurality of insertion sites,
each insertion site preferably formed by nucleic acid
encoding a specific restriction endonuclease site
('restriction site'). Together the restriction sites may
30 form a multiple cloning site (MCS) comprising a series of
overlapping or distinct restriction sites, preferably a

series of distinct restriction sites comprising one or
more of the ClaI, BglII, NruI, XhoI restriction sites.

The encoded components of the cassette may be arranged in
a predetermined order. In one arrangement, the one or
plurality of insertion sites is/are arranged upstream
(i.e. 5') of the ribosome binding site/first regulatory
sequence and the ribosome binding site/first regulatory
sequence is arranged upstream (i.e. 5') of the marker.

The first and second nucleotide sequences may comprise
nucleotide sequences having identity to regions of the
genome surrounding the insertion site in the selected
herpes simplex virus (the 'viral insertion site'). These
sequences enable the cassette to be incorporated at the
viral insertion site by homologous recombination between
the first and second nucleotide sequences and their
respective corresponding sequences in the viral genome.

Thus the first and second nucleotide sequences are
flanking sequences for homologous recombination with
corresponding sequences of a selected viral genome, such
homologous recombination resulting in insertion of the
cassette at the viral insertion site.

The first and second nucleotide sequences may correspond
to nucleotide sequences flanking an insertion site in the
RL1 locus of the HSV genome, more preferably in the
ICP34.5 protein coding sequence of the HSV genome.

The first and second nucleotide sequences may each be at
least 50bp in length, more preferably at least 100, 150,

200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200,
1300, 1400, 1500, 1600, 1700, 1800, 1900 2000, 2100,
2200, 2300, 2400, 2500, 2600, 2700, 2800, 2900, 3000,
3100, 3200, 3300, 3400, 3500, 3600, 3700, 3800, 3900 or

5   4000bp in length. Each of the first and second
         nucleotide sequences may have at least 50% sequence
         identity to their corresponding sequence in the viral
         genome, more preferably at least 60%, 70%, 75%, 80%, 85%,
         90%, 92%, 94%, 95%, 96%, 97%, 98% 99% or 100% identity.

10   Identity of sequences is determined across the entire
         length of a given nucleotide sequence. Where sequences
         are of different length, sequence identity of the shorter
         sequence is determined over the entire length of the
         longer sequence.

15

         The first and second nucleotide sequences may be
         characterised by the ability of one strand of a given
         sequence to hybridise with the corresponding single-
         stranded complement of the HSV genome under varying

20   hybridisation stringency conditions. Suitably, the first
         and second nucleotide sequences will hybridise with their
         corresponding complement under very low, low or
         intermediate stringency conditions, more preferably at
         high or very high stringency conditions.

25

         The viral insertion site is the position between the
         genomic nucleotide sequences corresponding to the first
         and second nucleotide sequences of the vector (the
         'genomic' and 'vector flanking sequences' respectively)

30   at which homologous recombination will occur and may be
         predetermined by selection of the vector flanking
         sequences. Where the genomic flanking sequences are

immediately adjacent, the insertion site is the position
between the peripheral and immediately adjacent bases of
the two genomic flanking sequences, such that insertion
of the cassette separates the genomic flanking sequences.
Where the genomic flanking sequences are separated by one
or a plurality of bases in the viral genome, the
insertion site is formed by said one or a plurality of
bases which are excised from the genome by the homologous
recombination event.

The position of the viral insertion site may be
accurately selected by careful selection and construction
of the vector flanking sequences. Accordingly, the
vector may be constructed such that homologous insertion
of the cassette results in disruption of a chosen protein
coding sequence and inactivation of the respective gene
product or such that the cassette is inserted at a non-
protein coding region of the viral genome. The complete
genome sequences of several herpes simplex virus strains
have been reported and are publicly available. The
complete genome sequence for HSV-1 strain 17syn+ was
reported by Dolan et al[3] (incorporated herein by
reference) and the complete genome sequence of HSV-2
strain HG52 was reported by Dolan et al[4] (incorporated
herein by reference) and is available from the EMBL
database under accession code Z86099. Using this
information, the vector of the present invention may
preferably be designed for use in generating mutant HSV-1
(e.g. in strain 17 or F) or mutant HSV-2 (e.g. in strain
HG52).

The first and second nucleotide sequences (vector flanking sequences) may each comprise sequence corresponding to the RL terminal repeat region of the genome of the selected HSV (e.g. HSV-1 strains 17 or F or

5   HSV-2 strain HG52). The vector flanking sequences may comprise, consist or consist essentially of nucleotide sequences of the RL repeat region which flank the ICP34.5 protein coding sequence. In flanking the ICP34.5 coding sequence, one or both of the selected sequences may, in

10   the corresponding HSV genome, overlap, i.e. extend into, the ICP34.5 protein coding sequence or one or both sequences may be selected so as to not overlap the ICP34.5 protein coding sequence. In a similar manner, the selected sequences may be chosen to overlap completely or

15   partially other important encoded signals, e.g. transcription initiation site, polyadenylation site, defined promoters or enhancers. In this preferred arrangement the insertion site will thus comprise all or a part of the ICP34.5 protein coding sequence and/or be

20   such that the inserted cassette disrupts the ICP34.5 protein coding sequence.

The vectors described, comprising first and second nucleotide sequences corresponding to regions of the RL

25   repeat region flanking and/or overlapping the ICP34.5 protein coding sequence, may be used in the generation of ICP34.5 null mutants wherein all or a portion of the ICP34.5 protein coding sequence is excised and replaced during the homologous recombination event such that both

30   copies of the ICP34.5 coding sequence are disrupted. The recombination may result in an insertion of nucleic acid within the ICP34.5 protein coding sequence thereby

disrupting that sequence. In that case, successfully
transformed virus are thus mutants incapable of
generating the ICP34.5 active gene product from at least
one copy, and preferably from both copies, of the ICP34.5

5   gene.

Successfully transformed virus are thus mutants incapable
of generating the ICP34.5 active gene product.

10   Each component of the cassette may be positioned
substantially adjacent the neighbouring component such
that a single bicistronic transcript comprising or
consisting essentially of the mRNA encoding the
nucleotide sequence of interest, ribosome binding site

15   and marker is obtainable.

The vectors described may further comprise, consist, or
consist essentially of a nucleic acid encoding a
selectable marker such as a polypeptide or protein

20   conferring antibiotic resistance e.g. kanamycin
resistance or ampicillin resistance.

The vectors described are preferably DNA vectors,
particularly dsDNA vectors. The vector may be provided as

25   a linear or circular (plasmid) DNA vector. The vector
preferably contains nucleotide sequences, e.g.
restriction endonuclease site(s), permitting transition
between the two forms by use of DNA ligation and
restriction materials (e.g. enzymes) and techniques known

30   to the person skilled in the art. To achieve homologous
recombination with a selected HSV, the vector is
preferably provided in linear form.

48

One such vector provided by the inventors is plasmid
RL1.dIRES-GFP deposited in the name of Crusade
Laboratories Limited having an address at Department of
5   Neurology Southern General Hospital 1345 Govan Road Govan
Glasgow G51 5TF Scotland on 03 September 2003 at the
European Collection of Cell Cultures (ECACC), Health
Protection Agency, Porton Down, Salisbury, Wiltshire, SP4
0JG, United Kingdom under accession number 03090303 in
10   accordance with the provisions of the Budapest Treaty on
the International Recognition of the Deposit of
Microorganisms for the Purposes of Patent Procedure
(herein referred to as the 'Budapest Treaty').

15   RL1.dIRES-GFP provides a platform for generating a
plurality of 'shuttle vectors' which can exploit the
process of homologous recombination to transfer a
nucleotide sequence of interest (downstream of a selected
promoter) into the disabling RL1 locus of HSV-1,
20   generating easily identifiable, oncolytic, ICP34.5 null
HSV-1 mutants expressing the products of the nucleotide
sequence of interest, e.g. an RNA transcript or a
polypeptide, and GFP. RL1.dIRES-GFP thus provides for
ease of generation and purification of ICP34.5 null HSV.
25

RL1.dIRES-GFP is a useful vector for making second-
generation oncolytic viruses having enhanced cytotoxic
potential and which may express the product(s) of
selected gene(s) to enhance the oncolytic and/or
30   therapeutic effect of the administered virus.

The RL1.dIRES-GFP plasmid incorporates a multi-cloning
sequence (MCS), upstream of an internal ribosome entry
site (IRES), the GFP gene and SV40 polyadenylation
sequences flanked by HSV-1 RL1 sequences. Incorporation

5   of the encephalomyocarditis virus IRES (EMCV IRES)
permits translation of two open reading frames from a
single transcribed mRNA.

Following generation of a specific shuttle vector by
10   cloning of the nucleotide sequence of interest (and the
selected promoter) into RL1.dIRES-GFP, recombinant HSV-1
expressing the desired nucleic acid transcript or
protein, can be generated and purified within 2 weeks.
This compares with 2-3 months using prior art protocols.
15

In the ICP34.5 null HSV generated using the RL1.dIRES-GFP
plasmid provided by the inventors transcription of both
the nucleotide sequence of interest and GFP as a single
transcript is controlled by the same promoter upstream of
20   the nucleotide sequence of interest, the transcribed IRES
directing cap-independent translation of GFP. The
generated ICP34.5 null HSV are non-neurovirulent. By
modifying the RL1.dIRES-GFP plasmid to incorporate
appropriate flanking sequences surrounding the cassette
25   other gene-specific HSV null mutants expressing GFP can
be generated.

RL1.dIRES-GFP is promoterless, thus enabling a promoter
of choice to be incorporated in the homologously
30   recombined shuttle vector for controlling expression of
the nucleotide sequence of interest from the inserted
cassette.

Plasmid RL1.dIRES-GFP or modified plasmid shuttle vectors
thereof further comprising nucleotide sequence encoding a
nucleic acid transcript or polypeptide of interest may be
5   provided in isolated or purified form.

The vector may be a variant of plasmid RL1.dIRES-GFP.

As the plasmid RL1.dIRES-GFP is designed for tandem
10   expression of a sequence of interest and the marker gene
encoding green fluorescent protein (GFP). The sequence of
interest is cloned into RL1.dIRES-GFP along with its
promoter (e.g. CMV) such that the promoter drives
transcription of an mRNA for the sequence of interest
15   along with the IRES-GFP. Translation results in
expression of the GFP from the internal ribosomal entry
site and the gene of interest and promoter must be cloned
into RL1.dIRES-GFP in the correct orientation to achieve
this. There are a number of instances where this tandem
20   expression arrangement may be unsuitable and a variation
of the cassette design is favourable.

One example is the expression of siRNAs as short hairpin
RNAs using RNA polIII promoters such as H1 or U6. These
25   promoters are unable to drive the additional tandem
expression of the IRES-GFP as the RNApolIII expression
cassette is designed only to produce short transcripts.
Additionally, sequences of interest derived from genomic
DNA with strong mRNA shut-off signals in their 3'
30   untranslated regions may not support IRES-GFP expression.

Thus in some cases a cassette may be provided in which
the sequence of interest and marker are expressed
separately from independent promoters.

5   One variant contains a cassette in which the ribosome
binding site of plasmid RL1.dIRES-GFP is replaced with a
regulatory nucleotide sequence, preferably a strong,
constitutive promoter such as the Phosphoglycerokinase
promoter. The marker is thereby expressed under the
10   control of this (the 'first') regulatory sequence. The
nucleotide sequence of interest (e.g. NTR, an antisense
or siRNA) is expressed under the control of a second
regulatory sequence upstream (5') of the nucleotide
sequence of interest, e.g. the CMV promoter. This vector
15   variant is particularly suitable for expression of siRNA
where a weak promoter may be used for expression of the
siRNA molecule or where the nucleic acid encoding the NTR
may have a strong termination signal making it difficult
to transcribe or translate a single bi- or poly-cistronic
20   transcript encoding the NTR and marker sequence. In this
arrangement the transformed virus containing the cassette
integrated in the viral genome produces two separate
transcripts under the control of the first and second
promoters.

25

One such cassette was constructed in the following
manner. The 1.3kbp blunt-ended EcoRI/AflII fragment that
contains the PGK promoter/GFP gene was obtained by
restriction digestion followed by Klenow treatment from
30   the vector pSNRG and cloned into the RL1-del vector cut
with the restriction enzyme NruI that generates blunt
ends. Successful insertion of the PGK/GFP DNA was

confirmed by BamHI digestion and the orientation of the
inserted DNA identified using the unique XhoI site in
RL1-del and the BsrGI site at the 3' end of PGK/GFP.
Plasmids with PGK/GFP in both forward and reverse

5   orientation were obtained and the plasmids were
designated RL1-dPGK/GFPfor and RL1-dPGK/GFPrev.
Expression of GFP was confirmed in BHK cells transfected
with the forward and reverse orientation plasmids.

10   Thus, sequences of interest along with their own
promoters (although it is preferred that the PGK promoter
is not also used for this purpose) can then be cloned
into either RL1-dPGK/GFPfor or RL1-dPGK/GFPrev in either
orientation using the remaining unique BglII, XhoI or

15   HpaI unique restriction enzyme sites. The resulting
plasmid can be used to derive recombinant HSV in which
the marker GFP gene and the gene of interest are
expressed independently from their own promoters.

20   The vectors described may be constructed for use in
generating engineered HSV-1 or HSV-2 by insertion of a
nucleic acid cassette through a mechanism of homologous
recombination between nucleotide sequences flanking the
cassette and corresponding sequences in the selected

25   herpes simplex virus genome.

The vectors described may comprise and have use as:
  i)   gene delivery (gene therapy) vectors for delivery
        of a selected nucleotide sequence, e.g. NTR, to a

30   specific locus of the HSV genome; and/or
  ii)   expression vectors for expression of the delivered
        nucleotide sequence of i) from the HSV genome

under the control of a selected regulatory

element; and/or

iii) vectors for the generation of HSV gene-specific

null mutants wherein the cassette is inserted at a

selected genomic location to disrupt the protein

coding sequence of a selected HSV gene such that

the gene product is inactive in the resultant

mutant virus.

The vectors described may be used in the manufacture of

engineered gene specific HSV null mutants, i.e. HSV

mutants incapable of expressing an active gene product of

a selected gene. They may be used in the manufacture of

engineered viruses which express a selected protein from

only one gene copy the other gene copy being disrupted or

modified such that it cannot express a functional gene

product. Such vectors may also be used in the manufacture

of a medicament, preferably comprising said gene specific

HSV null mutant, for use in treating cancer and tumours,

preferably by the oncolytic treatment of the tumour.

The vectors described may also be used in the manufacture

of engineered HSV mutants wherein the genome of the

mutant HSV comprises an exogenous or heterologous gene

which may have been inserted in the HSV genome by

homologous recombination of the cassette. Preferably, the

exogenous/heterologous gene is expressed in the mutant

HSV, which expression may be regulated by a regulatory

element, e.g. promoter, forming part of the inserted

cassette. Such vectors may be used in the manufacture of

a medicament, preferably comprising the engineered HSV

mutant, for use in the treatment of disease, including the oncolytic treatment of tumours.

The vectors described may also be used in the manufacture

5   of an engineered HSV mutant wherein the genome of the mutant HSV comprises an exogenous/ heterologous gene (i.e. a non-HSV originating gene) which may have been inserted in a protein coding sequence of the HSV genome by homologous recombination of the cassette such that the

10   mutant HSV is incapable of expressing the active gene encoded by said protein coding sequence and wherein the exogenous/ heterologous gene product is expressed under the control of a regulatory element. Preferably, the regulatory element forms part of the cassette. Such

15   vectors may be used in the manufacture of a medicament, preferably comprising the engineered HSV mutant, for use in the treatment of disease, including the oncolytic treatment of tumours.

20   The vectors described may also be used in the manufacture of an engineered HSV mutant wherein the genome of the mutant HSV comprises a nucleotide sequence which has been inserted in a protein coding sequence of the HSV genome by homologous recombination of the cassette such that the

25   mutant HSV is incapable of expressing the active gene encoded by said protein coding sequence and wherein the inserted nucleotide sequence is expressed under the control of a regulatory element to produce a desired transcript. Preferably, the regulatory element forms part

30   of the cassette. Such vectors may be used in the manufacture of a medicament, preferably comprising the

55

engineered HSV mutant, for use in the treatment of
disease, including the oncolytic treatment of tumours.

The vectors described may be used to generate mutant HSV
by inserting the cassette into the genome of a selected
HSV, the method of generation may comprise providing a
vector described above, where the vector is a plasmid,
linearising the vector; and co-transfecting a cell
culture with the linearised vector and genomic DNA from
said HSV.

The co-transfection may be carried out under conditions
effective for homologous recombination of said cassette
into an insertion site of the viral genome.

The method may further comprise one or more of the steps
of:

1) screening said co-transfected cell
culture to detect mutant HSV expressing
said marker; and/or

2) isolating said mutant HSV; and/or

3) screening said mutant HSV for expression
of the nucleotide sequence of interest
or the RNA or polypeptide thereby
encoded; and/or

4) screening said mutant HSV for lack of an
active gene product; and/or

5) testing the oncolytic ability of said
mutant HSV to kill tumour cells in
vitro.

Example 1

Construction of plasmid RL1.dIRES-GFP

5   General Approach

Plasmid RL1.dIRES-GFP was generated in three stages,
illustrated in Figure 1.

10   **1.** The DNA sequences containing the CMV IE promoter
(pCMV), the NAT gene, the internal ribosome entry site
(IRES), the GFP reporter gene and the SV40
polyadenylation sequences were excised from pNAT-IRES-GFP
using NsiI and SspI and purified.

15

2. The purified pCMV-NAT-IRES-GFP-PolyA DNA fragment was
cloned into RL1.del to form a new plasmid designated
RL1.dCMV-NAT-GFP.

20   **3.** The pCMV-NAT DNA sequences of RL1.dCMV-NAT-GFP were
excised using XhoI and the remainder of the plasmid re-
ligated to form a novel plasmid designated RL1.dIRES-GFP.
This novel plasmid contained a multi-cloning site (all
sites shown are unique) upstream of an IRES, the GFP gene
25   and the SV40 polyA sequences all within the HSV-1 RL1
flanking sequences. Recombinant ICP34.5 null HSV-1,
expressing a gene of interest in the RL1 locus, can be
generated by cloning the gene of interest (downstream of
a suitable promoter) into the multi-cloning site and co-
30   transfecting BHK cells with the plasmid and HSV-1 DNA.
Recombinant virus expressing the target gene can be
identified using GFP fluorescence.

Removal of the CMV promoter and noradrenaline transporter
gene (pCMV-NAT) from RL1.dCMV-NAT-GFP, followed by re-
ligation of the remainder of the plasmid, resulted in a

5   novel plasmid (RL1.dIRES-GFP) containing a multi-cloning
site (MCS), upstream of the encephalomyocarditis virus
internal ribosome entry site (EMCV IRES), the GFP
reporter gene and the SV40 PolyA sequences, all within
RL1 flanking sequences. This novel arrangement of DNA

10   sequences or 'smart cassette' allows ICP34.5 null HSV-1,
expressing a gene of interest in the RL1 locus, to be
easily generated by simply inserting the desired
transgene (downstream of a suitable promoter) into the
MCS and co-transfecting BHK cells with the plasmid and

15   HSV-1 DNA. The IRES situated between the GFP gene and the
MCS permits expression of two genes from the same
promoter and so recombinant virus expressing the gene of
interest also expresses GFP and can therefore be easily
identified under a fluorescence microscope and purified.

20

Materials and Methods

1μg of RL1.del* was digested with 10units HpaI (Promega)
in a suitable volume of 10x buffer (Promega) and nuclease

25   free water (Promega) at 37°C for 16hrs. The digested
plasmid was then purified using the QIAquick PCR
purification kit (Qiagen), treated with 10 units of Calf
Intestinal Phosphatase (Promega), in a suitable volume of
10x CIP buffer and nuclease free water for 4hrs at 37°C,

30   before being purified again using a Qiaquick PCR
purification kit. 5μl of the purified DNA was

electrophoresed on a 1% agarose gel to check its
concentration (Figure 2).

5   4 x 1µg of pNAT-IRES-GFP** was digested with 10 units of
       NsiI and 10 units of SspI in a suitable volume of 10x
       buffer (Promega) and nuclease free water (Promega) at 37⁰C
       for 16hrs. The reaction mixture was electrophoresed in a
       1% agarose gel for 1hr at 110 volts. The 5.4Kbp DNA
       fragment consisting of the CMV IE promoter (pCMV),
10   upstream of the noradrenaline transporter gene (NAT), the
       encephalomyocarditis virus internal ribosome entry site
       (IRES), the gene for green fluorescent protein (GFP) and
       the SV40 polyadenylation sequences (SV40 Poly A), was
       excised using a sterile scalpel and the DNA purified from
15   the gel using a QIAquick Gel Extraction kit (Qiagen). The
       eluted DNA was blunt ended using 3 units Klenow
       Polymerase (Promega) in accordance with the manufacturers
       instructions and the DNA purified using a QIAquick PCR
       purification kit (Qiagen). 5µl of the purified DNA
20   fragment was electrophoresed on a 1% agarose gel to check
       its concentration (Figure 3).

       Ligation reactions were carried out in small eppendorf
       tubes containing 5 units T4 DNA Ligase (Promega), a
25   suitable volume of 10X DNA Ligase Buffer (Promega),
       nuclease free water (Promega) and various volumes of the
       HpaI digested/CIP treated RL1.del and blunt ended pCMV-
       NAT-IRES-GFP-SV40 Poly A DNA, at 16⁰C overnight. Competent
       JM109 bacterial cells (Promega) were then transformed
30   with various aliqouts of the ligation reactions***.
       Colonies formed on the plates were picked, had their
       plasmid DNA extracted using a Qiagen Plasmid Mini kit and

screened for inserts using *Afl*II (New England Biolabs) restriction enzyme analysis. Plasmid DNA containing the insert would produce two fragments of 4.8Kbp and 9.2Kbp following digestion with *Afl*II. Two clones (clone 5 and

5   8) contained the insert (Figure 4). The orientation of the insert in clone 5 (RL1.dCMV-NAT-GFP) was determined using *Xho*I restriction enzyme analysis (Figure 5).

To generate RL1.dIRES-GFP from clone 5, the CMV-NAT

10   portion of the CMV-NAT-IRES-GFP-SV40 PolyA insert was removed by digesting 4 x 500ng of clone 5 with 10 units of *Xho*I in a suitable volume of buffer and water (Promega), overnight at 37^{0}C. The digested DNA was electrophoresed on a 1% agarose gel at 110 volts for 1hr

15   (Figure 6A). The 10.2Kbp fragment consisting of the IRES, the GFP gene, the SV40 PolyA sequences and RL1 flanking sequences in a pGEM3Zf(-) (Promega) backbone, was excised using a sterile scalpel and the DNA purified from the gel using a QIAquick Gel Extraction kit.

20

Ligation reactions were performed in small eppendorf tubes containing 100ng - 500ng purified DNA, 3 units T4 DNA Ligase (Promega), a suitable volume of 10X DNA Ligase Buffer (Promega) and nuclease free water (Promega)

25   overnight at 16^{0}C. Competent JM109 bacterial cells (Promega) were then transformed with various aliquots of the ligation reactions***. Colonies formed on the plates were picked, had their plasmid DNA extracted using a Qiagen Plasmid Mini kit and screened using *Xho*I (Promega)

30   restriction enzyme analysis. Colonies containing plasmid DNA from which CMV-NAT had been removed would produce one fragment of 10.2Kbp when digested with *Xho*I. Several

positive clones were found, one was isolated, and a
large-scale plasmid preparation undertaken using
Promega's Wizard Plus Maxipreps kit. The large-scale
plasmid preparation was checked by digesting with *XhoI*
5   (Figure 6B). This plasmid DNA was subsequently named
'RL1.dIRES-GFP'.

Plasmid RL1.dIRES-GFP has been deposited in the name of
Crusade Laboratories Limited having an address at
10 Department of Neurology Southern General Hospital 1345
Govan Road Govan Glasgow G51 5TF Scotland on 03 September
2003 at the European Collection of Cell Cultures (ECACC),
Health Protection Agency, Porton Down, Salisbury,
Wiltshire, SP4 0JG, United Kingdom under accession number
15 03090303 in accordance with the provisions of the
Budapest Treaty.

RL1.del
*RL1.del was provided by Dr.E.McKie and is the pGEM-3Zf(-
20) plasmid (Promega) into which has been cloned an HSV-1
fragment (123459-129403) consisting of the RL1 gene and
its flanking sequences. The 477bp *Pfl*MI-*Bst*EII fragment
of the RL1 gene (125292-125769) has been removed and
replaced with a multi-cloning site (MCS) to form RL1.del.
25

pNAT-IRES-GFP
** pNAT-IRES-GFP was supplied by Dr. Marie Boyd (CRUK
Beatson Laboratories) and is the pIRES2-EGFP plasmid (BD
Biosciences Clontech) into which she has cloned the
30 bovine noradrenaline transporter (NAT) gene (3.2Kbp), at
the *Nhe*I and *Xho*I sites.

***Transformation of Bacterial Cells*

10µl of a glycerol *E.coli* stock was added to 10ml 2YT

medium in a 20ml griener tube. This was placed in a 37°C

shaking incubator for 16-24hrs until a saturated culture

5   was obtained. 1ml of this culture was then added to

100ml of 2YT in a 500ml sterile glass bottle and placed

in the 37°C shaking incubator for 3hrs. The bacterial

cells were pelleted by centrifugation at 2,000rpm for 10

minutes (Beckman). The cells were then resuspended in

10   1/10ᵗʰ volume of transformation and storage buffer (10mM

$MgCl_2$, 10mM $Mg(SO)_4$, 10% (w/v) PEG 3,500, 5% (v/v) DMSO).

The cells were placed on ice for between 10 minutes and

2hrs, after which time they were considered competent for

transformation.

15

1-10µl of DNA was mixed with 100µl of competent bacteria

in eppendorf tubes, and the tubes placed on ice for 30

minutes. After this, the samples were 'heat shocked' by

incubating the tubes in a 42°C water bath for exactly 45

20   seconds before placing them on ice for a further 2

minutes. 1ml of L-Broth was added, the tube inverted 2-3

times, and the bacteria incubated for 1hr at 37°C. 100µl

of the transformed bacteria was plated out onto L-broth

agar plates containing 100µg/ml of the appropriate

25   antibiotic (usually ampicillin or kanamycin). Plates

were allowed to dry at room temperature, before

incubating in an inverted position at 37°C overnight.

Example 2

30

Generation of ICP34.5 null HSV-1 expressing a gene
product of interest and GFP using plasmid RL1.dIRES-GFP.

General Approach

Generation of ICP34.5 null HSV-1 expressing a gene
5   product of interest requires insertion of nucleotide
sequence encoding the gene product (polypeptide) of
interest, and often a desired promoter, at the MCS of
RL1.dIRES.GFP followed by co-transfection of BHK cells
with the linearised plasmid, containing the gene of
10   interest, and HSV DNA. Following homologous recombination
viral plaques expressing GFP are identified. Figure 7
illustrates the method steps involved.

Referring to Figure 7A plasmid DNA, containing the gene
15   of interest and the desired promoter (X), is digested
with restriction endonucleases to release the
promoter/gene fragment.

The promoter/gene fragment is purified and cloned into
20   the multi-cloning site (MCS) of RL1.dIRES.GFP forming a
shuttle vector suitable for generating oncolytic HSV-1
(Figure 7B). This vector contains HSV-1 sequences that
flank the essential RL1 gene but does not contain the RL1
gene. The plasmid also contains the gene for Green
25   Fluorescent Protein (GFP) downstream of an internal
ribosome entry site (IRES). The IRES permits expression
of both the gene of interest and the GFP gene from the
same upstream promoter.

30   BHK cells are then co-transfected with linearised
RL1.dIRES.GFP, now containing the gene of interest, and
HSV-1 DNA (Figure 7C). Following homologous

recombination, designer virus, expressing the gene of
interest and GFP, is generated and can be distinguished
from wild type virus (also generated but not expressing
GFP) under a fluorescence microscope.

Viral plaques, expressing GFP (and hence the gene of
interest), are picked under the fluorescence microscope
and purified until all wild-type HSV-1 has been removed.
The recombinant HSV-1 is considered 100% pure when all
the viral plaques are expressing GFP (Figure 7D).

Once the recombinant virus is completely pure, an
isolated plaque is picked and a highly concentrated stock
is grown and titrated (Figure 7E). Oncolytic HSV-1,
expressing a gene product of interest from a selected
promoter, is then ready for characterisation and in vitro
examination of its tumour killing potential.

Materials and Methods

To generate recombinant ICP34.5 null HSV-1 expressing a
gene of interest and GFP, requires the gene of interest,
and often a suitable promoter, to be cloned into the MCS
of RL1.dIRES-GFP in the forward orientation with respect
to the GFP gene in this plasmid. Once this has been
achieved the plasmid is linearised (i.e. digested with a
restriction enzyme that cuts only once, usually SspI or
ScaI) in an irrelevant region. 80% confluent BHK cells in
60 mm petri dishes are then co-transfected with HSV-1 DNA
and linearised plasmid DNA as described below.

To generate replication restricted HSV-1, expressing the gene of interest and GFP, the gene of interest must be cloned into RL1.dIRES-GFP downstream of a suitable promoter (e.g. CMV IE). The promoter is required upstream

5   of the gene of interest for the production of a bicistronic mRNA transcript. The IRES sequence between the two open reading frames in the transcript functions as a ribosome binding site for efficient cap-independent internal initiation of translation. The design enables

10   coupled transcription of both the gene of interest and GFP, followed by cap-dependent initiation of translation of the first gene (gene of interest) and IRES-directed, cap-independent translation of GFP. Co-ordinate gene expression is thus ensured in this configuration.

15

Co-Transfection of Virus and Plasmid DNA by CaPO$_4$ and DMSO Boost

HSV-1 (17$^+$) DNA and 0.1-1µg linearized SMART cassette containing the gene and promoter of interest is pipetted

20   into 1.5ml eppendorf tubes containing 1µl of calf thymus DNA (10µg/ml) and an appropriate volume of distilled water to give a final volume of 165µl. The solutions are very gently mixed using a 200µl pipette tip. 388µl of HEBS, pH 7.5, (130mM NaCl, 4.9mM KCl, 1.6mM Na$_2$HPO$_4$, 5.5mM

25   D-glucose, 21mM HEPES) is then added, the solution mixed, before adding 26.5µl of 2M CaCl$_2$ dropwise and flicking the eppendorf tube two or three times. The samples are left at room temperature for 10-15 minutes then added dropwise to 80% confluent BHK's in 60mm petri dishes from which

30   the medium has been removed. Following incubation at 37°C for 45 minutes, the cells are overlaid with 5ml of ETC10

and incubated at 37°C. Three to four hours later, the
media is removed and the plates washed with ETC10. For
exactly 4 minutes, the cells are overlaid with 1ml 25%
(v/v) DMSO in HEBS at room temperature. After the 4
5   minutes, the cells are immediately washed three times
with 5ml ETC10 before overlaying with 5ml of ETC10 and
returning to the incubator. The following day, fresh
medium is added to the cells. Two days later, when cpe
is evident, cells are scraped into the medium,
10   transferred to small bijoux and sonicated thoroughly.
The sample is then stored at -70°C until required (see
section below on plaque purification).

N.B. The volume of virus DNA to add is determined by
15   undertaking the above procedure without plasmid DNA,
using a range of virus DNA volumes and choosing the
volume that gives the greatest number of viral plaques on
the BHK monolayer after 2 or 3 days.

20   *Plaque Purification*
Sonicated samples from co-transfection plates are thawed
and serially diluted 10 fold in ETC10. 100µl from neat to
the 10^5 dilution is plated out on confluent BHK's in 60 mm
petri dishes from which the media has been removed.
25   After 45 minutes incubation at 37°C, the cells are
overlaid with 5ml EMC10 and incubated at 37°C for 48hrs.
The plates are then checked for the presence of viral
plaques and those dishes with the fewest, most separated
plaques are placed under a fluorescent stereomicroscope.
30   Recombinant virus, designed to express the green
fluorescent protein (GFP) in addition to the gene of
interest, can clearly be distinguished from wild type

virus using a GFP filter. Fluorescent plaques are picked
using a 20µl pipette and placed (including the tip) into
an eppendorf tube containing 1ml ETC10. The sample is
thoroughly sonicated before making serial 10 fold
5   dilutions in ETC10 and repeating the above purification
procedure. The process is repeated typically 3-4 times
until every plaque on the BHK monolayer is fluorescent.
Once this has been achieved, 50µl of this sample is used
to infect BHK cells in roller bottles, in 50ml ETC10, and
10   a virus stock grown.

Tissue Culture Media
BHK21/C13 cells are grown in Eagle's medium (Gibco)
supplemented with 10% newborn calf serum (Gibco) and 10%
15   (v/v) tryptose phosphate broth. This is referred to as
ETC10. For virus titrations and plaque purification,
EMC10 (Eagles medium containing 1.5% methylcellulose and
10% newborn calf serum) is used to overlay the cells.

20   **Example 3**

Construction of HSV1716/CMV-NTR/GFP

General Approach

25

HSV1716/CMV-NTR/GFP was generated by cloning a 1.6Kbp
*Bam*HI fragment from pPS949[10], consisting of the *E.coli*
nitroreductase (NTR) gene downstream of the CMV IE
promoter (pCMV), into the MCS of the RL1.dIRES-GFP smart
30   cassette, in the forward orientation with respect to the
GFP gene in RL1.dIRES-GFP (Figure 8). The resultant
plasmid, named RL1.dCMV-NTR-GFP, was then linearised and

recombinant virus generated and purified as described
above. The plasmid pPS949 (referred to as 'pxLNC-ntr' in
Ref 10) containing the NTR gene downstream of the CMV IE
promoter (pCMV-NTR) in a pLNCX (Clontech) backbone, was a
5   kind gift from Professor Lawrence Young, University of
Birmingham, UK.

Materials and Methods

10   4 x 1µg of pPS949 was digested with 10 units of *Bam*HI
(Promega), in a suitable volume of 10x buffer (Promega)
and nuclease free water (Promega), at 37°C for 16hrs. The
reaction mixture was electrophoresed in a 1% agarose gel
for 1hr at 110 volts. The 1.6Kbp DNA fragment consisting
15   of the CMV promoter upstream of the NTR gene (pCMV-NTR),
was excised using a sterile scalpel and the DNA purified
from the gel using a QIAquick Gel Extraction kit
(Qiagen). 5µl of the purified DNA fragment was
electrophoresed on a 1% agarose gel to check its
20   concentration (Figure 9).

2µg of the RL1.dIRES-GFP smart cassette was then digested
with 15 units of *Bgl*II (Promega), in a suitable volume of
10x buffer (Promega) and nuclease free water (Promega),
25   at 37°C for 16hrs. The digested plasmid was then purified
using the QIAquick PCR purification kit (Qiagen), treated
with 10 units of Calf Intestinal Phosphatase (Promega),
in a suitable volume of 10x CIP buffer and nuclease free
water for 4hrs at 37°C, before being purified again using
30   the Qiaquick PCR purification kit. 5µl of the purified
DNA was electrophoresed on a 1% agarose gel to check its
concentration (Figure 10).

Ligation reactions were carried out in small eppendorf
tubes containing 5 units T4 DNA Ligase (Promega), a
suitable volume of 10X DNA Ligase Buffer (Promega),

5   nuclease free water (Promega) and various volumes of the
BglII digested/CIP treated RL1.dIRES-GFP smart cassette
and pCMV-NTR (BamHI ends), at 16°C overnight. Competent
JM109 bacterial cells (Promega) were then transformed
with various aliqouts of the ligation reactions. Colonies

10   formed on the plates were picked, had their plasmid DNA
extracted using a Qiagen Plasmid Mini kit and screened
for inserts using BglII/XhoI (Promega) restriction enzyme
analysis. RL1.dIRES-GFP plasmid DNA containing the pCMV-
NTR insert in the correct orientation would produce two

15   fragments of 11.5Kbp and 300bp following digestion with
BglII and XhoI. One clone (clone 4) was found to contain
the insert in the correct orientation (Figure 11). This
plasmid was named 'RL1.dCMV-NTR-GFP'.

20   0.1-1µg of RL1.dCMV-NTR-GFP was linearized by digesting
with 10 units of ScaI (Promega), in a suitable volume of
10x buffer (Promega) and nuclease free water (Promega),
at 37°C for 16hrs. A sample (5µl) of the digested DNA was
electrophoresed on a 1% agarose gel for 1hr at 110 volts

25   to check that it had been linearized. 80% confluent BHK
cells were then co-transfected with a suitable volume of
the remaining linearised DNA and HSV-1 DNA. Recombinant
HSV-1, expressing GFP (and hence NTR), was identified and
purified using a fluorescent microscope and a virus

30   stock, named HSV1716/CMV-NTR/GFP, was grown and titrated
on BHK cells (Figure 12).

HSV1716/CMV-NTR/GFP has been deposited in the name of
Crusade Laboratories Limited having an address at
Department of Neurology Southern General Hospital 1345
Govan Road Govan Glasgow G51 5TF Scotland on 05 November
5   2003 at the European Collection of Cell Cultures (ECACC),
Health Protection Agency, Porton Down, Salisbury,
Wiltshire, SP4 0JG, United Kingdom under accession number
03110501 in accordance with the provisions of the
Budapest Treaty.

10

HSV1716/CMV-NTR/GFP Cell Killing

HSV1716/CMV-NTR/GFP replicates with almost identical
kinetics to HSV1716 in BHK cells and 3T6 cells. BHK cells
15   support the replication of ICP34.5 null HSV while
confluent 3T6 cells do not. Figure 13 shows that
HSV1716/CMV-NTR/GFP will replicate as well as HSV1716 in
permissive cell lines and that the introduction of
exogenous genes, e.g. NTR and GFP, has not reduced the
20   oncolytic potential of the ICP34.5 null HSV. The fact
that HSV1716/CMV-NTR/GFP fails to replicate in 3T6 cells
also indicates that this recombinant HSV is an ICP34.5
null mutant.

25   Figure 14 is a Western blot demonstrating that no ICP34.5
polypeptide is expressed from HSV1716/CMV-NTR/GFP, and
that the virus is thus useful as a gene therapy vector.

Figure 15 is another Western blot demonstrating
30   expression of NTR in a variety of cell lines infected
with HSV1716/CMV-NTR/GFP, including a human malignant
melanoma cell line (C8161) and confluent 3T6 cells in

which ICP34.5 null HSV does not replicate. Expression of
NTR in confluent 3T6 cells, following infection with
HSV1716/CMV-NTR/GFP, is encouraging as it demonstrates
that replication of this ICP34.5 null mutant is not

5   required for expression of the prodrug-activating gene
(i.e. NTR). Some tumour cells *in vivo* will not support
the replication of ICP34.5 null HSV and as such, will not
be killed with HSV1716.

10   Figure 16 shows the results from a cytotoxicity assay
performed in confluent 3T6 cells. Infecting confluent 3T6
cells with an ICP34.5 null mutant (HSV1716/CMV-NTR/GFP),
at a multiplicity of infection (MOI) of 1 plaque forming
units (pfu)/cell, does not result in any significant cell

15   death, neither does separate incubation of the cells with
50μM CB1954. However, significant cell death is evident
72hrs post infection with 1pfu/cell HSV1716/CMV-NTR/GFP
when 50μM CB1954 is included in the growth medium. This
clearly demonstrates that when there is no replication of

20   the virus, substantial cell death is still possible from
virus directed enzyme prodrug therapy (VDEPT).

Infecting confluent 3T6 cells with an ICP34.5 null mutant
at a MOI of 10pfu/cell will result in cell death, by a

25   mechanism known as 'viral antigen overload'. However, the
level of cell killing is even more pronounced
(approximately 20% more), when 50μM CB1954 is included in
the growth medium.

30   A similar cytotoxicity assay was performed in human C8161
melanoma cells, the results are set out in Figure 17.
Unlike confluent 3T6 cells, C8161 cells do support the

replication of ICP34.5 null HSV. Therefore, cell death
will occur following infection of the cells with ICP34.5
null HSV, at 1pfu/cell. However, when CB1954 is included
in the overlay of HSV1716/CMV-NTR/GFP infected cells, the

5   cells are killed more efficiently and more quickly. No
enhanced cell killing is evident when CB1954 is included
in the overlay of cells infected with HSV1716-GFP. These
results demonstrate that enhanced cell killing is
possible in human tumour cells.

10

Cell culture images for the cytotoxicity assays performed
in confluent 3T6 and human C8161 melanoma cells are shown
in Figures 18 and 19.

15   **Example 4 - In vivo evaluation of the anti-tumour
activity of a selectively replication competent herpes
simplex virus in combination with enzyme pro-drug
therapy.**

20   The anti-tumour activity of a selectively replication
competent herpes simplex virus in combination with an
enzyme prodrug therapy approach in appropriate animal
models *in vivo* was investigated.

25   The parental virus, HSV 1716 is a selectively replication
competent mutant of the herpes simplex virus 1 (HSV 1)
which lacks both copies of the RL1 gene that encodes the
protein ICP 34.5. This protein is a specific determinant
of virulence. The function of this protein has been

30   described at length elsewhere[12]. The virus can grow only
in cells that have a high level of functional PCNA. High

levels of PCNA are found only in cells that are dividing such as tumour cells and not normal differentiated cells.

It has already been shown that HSV 1716 can achieve
5   selective tumour cell killing with minimal toxicity and improved survival times in a number of animal models[13]. Initial phase 1 clinical trials using HSV1716 virus in patients has also meet with some success[14, 15].

10   Although HSV1716 selectivity replicates in tumour reducing the tumour bulk by cell lysis the inventors did not anticipate HSV1716 to lytically replicate in all cells in the tumour due to the heterogeneity of the cell type and growth state.
15

In order to enhance the efficacy of the tumour cell killing – hence kill the entire tumour – the inventors have constructed a derivative of HSV1716 designated HSV1716/CMV-NTR/GFP that expresses the E.Coli
20   nitroreductase gene (ntr) under the control of a CMV early promoter (see example 3 above). In this example and the figures referred to HSV1716/CMV-NTR/GFP is called HSV1790.

25   The enzyme ntr converts the inactive prodrug CB1954 to a functional cytotoxic alkylating agent that kills both dividing and non-dividing cells by apoptosis. This active drug is diffusible and membrane permeable resulting in an efficient bystander effect, i.e. wherein the activated
30   drug may have an effect on surrounding cells.

As the prodrug will only be converted to its active form
in the tumour which has been infected with *ntr* expressing
virus, toxicity to normal cells is avoided hence
improving the therapeutic index following systemic

5   delivery of this compound.

Initial *in vitro* experiments using this combination have
already shown enhanced cell kill using this virus in
combination with CB1954 in a number of cell lines.

10

This example further evaluates this combination approach
in vivo in appropriate animal models.

Results

15

Months 1 - 3

Months 1 - 3 were taken up mainly by *in vitro* work.
During this time period high titre, sterile virus stock

20   was generated for use in the xenograft models.

Xenograft models were also generated in athymic nude mice
using the cell line A2780, a human ovarian epithelial
carcinoma line initially derived from a tumour sample

25   from an untreated patient (European Collection of Cell
Cultures (ECACC) CAMR, Porton Down, Salisbury, Wiltshire,
SP4 0JG, United Kingdom, accession number 93112520).

Generation of a gliomal xenograft model was attempted

30   using 2 gliomal lines that were available in house, LN-18
and U373MG. There are reports in the literature of both
being successfully grown as xenografts in athymic mice.

74

However, as shown in the table below the inventors failed to see any xenograft growth up to 28 days after injection with 5 million cells subcutaneously.

5   **Table 1**

Cell Line	No of cells injected per mice	Number of mice	Presence of xenograft 28 days after cell injection
LN 18	5 million	5	0/5
U373 MG	5 million	5	0/5

A2780 tumour take

10

As reported previously A2780 have a Take Rate of approximately 50 % - that is 50 % of mice that are injected with 5 million cells per flank subcutaneously will develop xenografts. When the number of cells

15   injected was increased to 10 million or more an increase in take rate of approximately 15 - 25 % was seen, giving an overall take rate of 65 - 75 %.

Thus increasing the number of gliomal cells injected may

20   increase the take rate of these cell lines

Dose response to the HSV 1790 virus

Before the mice can be treated with a combination of

25   virus and prodrug, first one must carry out experiments

to make informed decisions about how much of the virus,
and the prodrug to give.

A dose response experiment will allow one to find both
5   the most appropriate does of the virus to use in the
experiments and the maximum tolerated dose (MTD) of the
virus, that is, the largest amount of the virus that can
be given to a single mouse without adverse side effects.
Small groups of tumour bearing mice are given a small
10   dose of virus. Assuming they do not have any adverse
effects another group is given a larger dose of virus.
This continues until either the mice start to suffer ill
effects or we reach a maximum dose.

15   The maximum amount of virus that can be intratumourally
injected is 100 μl, hence the maximum dose from our
current stock is 10^9 PFU per injection.

Figure 20 shows the weight change in the mice after
20   injection with a variety of doses of virus. Weight is a
good indicator of the animals overall health. Any loss of
weight signifies that the treatment is not being well
tolerated. Where an animal loses more than 20% of its
initial body weight it was sacrificed immediately.
25
A dose of 10^9 PFU of the HSV 1790 virus is not tolerated
by these mice, they rapidly lost body weight and were
sacrificed at Day 3 post injection. Doses of 10^8 PFU or
less were better tolerated, the mice initially lost
30   weight in the days following injection but quickly
recovered to approximately their initial body weights.

It should be pointed out that as the experiment
progresses the animals appear to be increasing in weight.
This is almost certainly due to the fact that it is total
body weight that is measured, which includes the weight
5   of any tumour that is forming.

Response of the tumour to HSV 1790 treatment

Tumour volume was measured daily after intratumoural
10   injection of the HSV 1790 virus to look for any growth
delay or regression of the tumours.

Figure 21 shows the change in tumour volume as measured
over a period of 100 days. If the tumour was injected
15   with PBS only as a control the tumour increased in size
rapidly and by approximately Day 13 post injection the
tumours had become too large and the animals had to be
sacrificed.

20   Treatment with all doses of virus appeared to delay the
growth of the tumour to some degree. Doses of 10^5 PFU
increased the longevity of the mice by approximately 12
days while mice injected with 10^6 PFU virus tumours
survived for an extra 23 days compared to the control
25   group before the tumours became prohibitively large.
Perhaps surprisingly the group of mice injected with 10^8
PFU of virus survived only slightly longer than the
control group. It is possible there were a large number
of non infectious particles or that sheer number of
30   particles caused the cells which the virus would have
grown in to be killed.

The group of mice treated with 10^7 PFU of virus survived
the longest and indeed two out of three of the mice did
not have any visible signs of tumour when sacrificed at
day 100.

5

Naked DNA Experiments

In order to check the alterations in tumour growth are
due to the virus itself and not a result of the CMV-ntr
10   plasmid DNA that had been introduced to the HSV 1716
virus, an experiment was set up looking at the effect of
the CMV-ntr plasmid DNA alone and in combination with the
prodrug CB1954.

15   Mice were randomised into treatment groups of 6 animals
each when tumour diameters are approximately 5mm (this is
Day 0). Figure 22 shows the starting tumour diameters for
the mice used in this experiment. Two groups of mice were
administered CMV-ntr plasmid by direct intratumoural
20   injection at a dose of 0.2mg DNA per injection. One of
these groups was then administered with a single dose of
80mg/kg of CB1954 on Day 2 by intra-peritoneal injection.
The third group of mice had a single administration of
CB1954 (80mg/kg) by intra-peritoneal injection on Day 2
25   following intratumoural injection of saline control at
Day 0. Animals were weighed daily (Figure 23) and daily
caliper measurements performed until the tumour sizes
were in the region of 20mm by 20mm. Tumour volumes were
estimated from these measurements (volume = d3 x 6)
30   (Figure 24). In addition any toxicity from these
administered agents was determined. On the basis of these
experiments the inventors determined that neither the

CMV-ntr alone, CB1954 alone or the combination of both
CMV-ntr and CB1954, has any anti - tumour activity as
determined by tumour regression in this model system
(Figure 24).

Scheduling Experiment

Previous dose response experiments have shown that doses
of less than 10^8 PFU virus per mouse do not appear to have
any adverse effect of the animals health.

A dose of 10^7 PFU virus per mouse resulted in a great
reduction in tumour growth, indeed after 100 days two out
of three of the mice in the group had no visible tumour.
This is very encouraging - the virus only at high doses
may be enough to delay growth or cause tumour regression.

However to look at the effect of a combination of the
virus and the prodrug CB 1954 a lower dose of the virus
was studied - if the treatment of the virus alone results
in growth delay for such a long period one would be
unable to ascertain the addition or synergistic effects
of the prodrug.

The inventors proceeded to investigate two doses of the
virus in combination with CB1954. The doses selected
were 10^5 PFU and 10^6 PFU. Both these doses caused some
tumour growth delay in earlier experiments.

The prodrug is given as an 80mg/kg intra-peritoneal
injection, after dissolving the powdered form in 10%
acetone and then making up the volume with peanut oil.

Another factor that determines how well the drug will
work - hence how much tumour growth delay or regression
is seen - is when the drug is actually given. As the
5   prodrug will only be converted to an active substrate in
the presence of NTR it was considered that the virus
containing the NTR will have to been given first. It was
also considered that if the virus is given time to
replicate and produce more NTR then the prodrug may have
10 a more pronounced effect.

To discover the optimal doses of both the virus and the
drug and the optimal times of these treatments a
scheduling experiment was set up.
15
Mice were randomized into treatment groups (treatment
regimes shown in Table 2) of 3 animals when tumour
diameters were approximately 5mm (tumour volume 0.5 - 1.5
mm^3). Figure 25 shows the starting tumour volumes of each
20 of the groups.

Table 2: Treatment groups

1	10^5 HSV 1790 + drug (Day 2) + Drug (Day10)
2	10^5 HSV 1790 + drug (Day 2) + vehicle (Day 10)
3	10^5 HSV 1790 + vehicle (Day 2) + drug (Day 10)
4	10^5 HSV 1790 + vehicle (Day 2) + vehicle (Day 10)
5	No virus + drug (Day 2) + drug (Day 10)
6	No virus + drug (Day 2) + vehicle (Day 10)
7	No virus + vehicle (Day 2) + drug (Day 10)
8	No virus + vehicle (Day 2) + vehicle (Day 10)
	·
9	10^6 HSV 1790 + vehicle (Day 2) + vehicle (Day 10)
10	10^6 HSV 1790 + drug (Day 2) + vehicle (Day 10)
11	10^6 HSV 1790 + drug (Day 2) + drug (Day 10)
12	10^6 HSV 1790 + drug (Day 2) + drug (Day 10) + drug (Day 15)
13	No virus + drug (Day 2) + drug (Day 10)
14	No virus + vehicle (Day 2) + vehicle (Day 10)
15	10^5 HSV 1716 + drug (Day 2) + drug (Day 10)
16	10^5 HSV 1716 + vehicle (Day 2) + vehicle (Day 10)
17	10^5 HSV 1716 + drug (Day 2) + vehicle (Day 10)
18	10^5 HSV 1716 + vehicle (Day 2) + drug (Day 10)

The treatment groups were administered with a single
direct intratumoural injection of the virus and dose
determined for that group. The virus was diluted PBS +
10% serum. 'No virus' control groups received an

5   intratumoural injection of 100μl of PBS + 10% serum. This
day was designated as Experimental Day 0.

The intratumoural injections did not appear to have any
adverse effects on the mice. Some tumours bleed slightly

10   after injection but not to a great degree. The animals
did not lose body weight (Figure 26) and their behaviour
did not appear to alter. In all the tumours that bleed
slightly, the following day the healing process had begun
and within 3 - 5 days there was little evidence of any

15   puncture wound on any tumour.

Injections of CB1954 were given to the appropriate groups
at days 2, 10 and 15. A dose of 80mg/kg - the equivalent
of approx. 2mg per mouse - was given. The powdered form

20   of the CB1954 drug (from Sigma) was dissolved in acetone
to 10% of the final volume (10μl per 2mg). The volume was
then made up to 2mg CB1954 in 100μl using peanut oil. A
syringe was used to mix the drug as peanut oil is thick
and viscous. The drug was made up fresh every time.

25
The appropriate groups were then injected intra-
peritoneal with this solution. Control groups which were
not receiving drug were injected intra-peritoneal with a
100μl solution of 10% acetone in peanut oil.

30
No swelling or irritation at the site of injection was
noted on any of the mice either at time of injection or

at any later time point. The mice appeared slightly
lethargic for a short period after the injection but did
not lose any body weight (Figure 26) or show signs of
lethargy the following day.

5

No virus + CB1954 prodrug

Groups 5,6,7,8, 13 & 14 looked at the effect of prodrug
alone on tumour growth. Figure 27 shows that there is

10   little effect on tumour growth when CB1954 is given
alone.

10^5 PFU virus +/- CB1954 prodrug

15   10^5 PFU virus was given at Day 0 followed by either
prodrug or vehicle at Days 2 and 10.

As can be seen from the graph in Figure 28 tumours
treated with either virus only or virus and prodrug did

20   not grow as large as the untreated tumour. The tumour
treated with the virus grew only to approximately half
the size of the untreated control.   .

Treatment with virus and prodrug resulted in tumours

25   which grew to only approx 2 - 3 mm^3 in volume. This is
significantly less than the untreated tumours which grew
in size to approx 20 mm^3.

10^6 PFU virus +/- CB1954 prodrug

30

Figure 29 shows the changes in tumour volume over time
after treatment with a higher dose of virus, 10^6 PFU per

injection, in combination with the prodrug, given as
described in Table 2. As with the lower virus dose,
treatment with either virus only, or in combination with
CB1954, results in significantly smaller tumours compared

5   to the untreated controls.

HSV 1716 virus in combination with CB1954 prodrug

The parental strain of the virus, which has not been

10   engineered to contain the CMV-ntr DNA was examined for
its effects on tumour growth delay. This virus does have
an oncolytic effect, however it doesn't contain the NTR
gene needed to convert the inactive prodrug into its
active metabolite. Therefore one would not expect any

15   additional or synergistic effects when the prodrug is
added in combination with the virus. Figure 30 shows the
results of this experiment.

The combination of the virus and the prodrug appeared to

20   produce some reduction in tumour growth compared to the
untreated control tumours.

The groups used in these results contained only 2 or 3
animals. The animals used were also older and their

25   tumours had taken longer to grow than those used in
previous experiments. Hence it is possible that repeating
the experiment with a larger number, with younger mice or
quicker forming tumours may result in a more marked
growth delay after treatment with the HSV 1716 virus.

30

*Comparison of HSV 1790 (at 10^5 and 10^6) and HSV 1716 in
combination with CB1954 prodrug*

Figure 31 shows a comparison between the two doses of the
HSV 1790 virus in combination with the prodrug and the
HSV 1716 prodrug combination. The parental virus HSV

5   1716 shows some growth delay in comparison with the
untreated control. We would assume that this growth delay
is due to the oncolytic effect of the virus as the NTR
gene is not present to alter the inactive prodrug into
its active form.

10

Tumour growth is reduced further when the tumour is
treated with the HSV 1790 virus containing the NTR gene.
This appears to be dose dependent – the higher dose of
the virus results in a greater growth delay than the

15   lower dose.

In conclusion it would appear from these results that
indeed the HSV 1790 virus used in combination with the
prodrug CB1954 results in growth delay in the model

20   system examined. Giving both virus and drug in
combination has a greater effect than given either alone.

It appears that the timing at which the prodrug is given
after virus treatment is important. When CB1954 was given

25   soon after viral injection (Day 2 post viral injection)
tumour growth was not delayed as much as if the drug was
given at a later date (Day 10). It may be that given at
Day 2 the drug killed the cells that were supporting
viral growth and replication and actually reduced the

30   oncolytic effect of the virus.

By day 10 the virus may have replicated and killed as
many cells by oncolysis as possible. It is anticipated
that due to heterogeneity of the cell type and growth
state that all the cells within a tumour would not be
5   susceptible to lysis by the virus. The drug then comes
in and 'mops up' by killing any cells that are supporting
viral growth (hence containing the NTR gene) but were not
susceptible to oncolysis. As the active drug is
diffusible and membrane permeable it may have a bystander
10   effect - killing not only the cells infected with the
virus but also its near neighbours.

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